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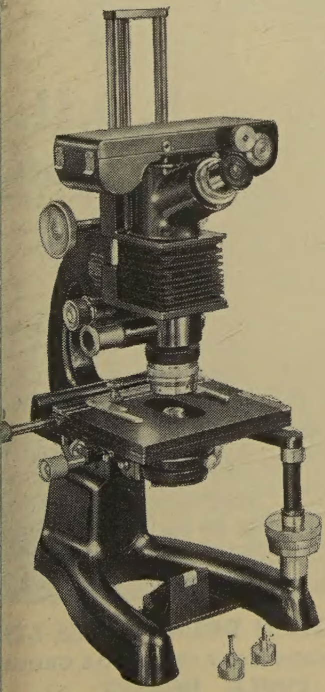

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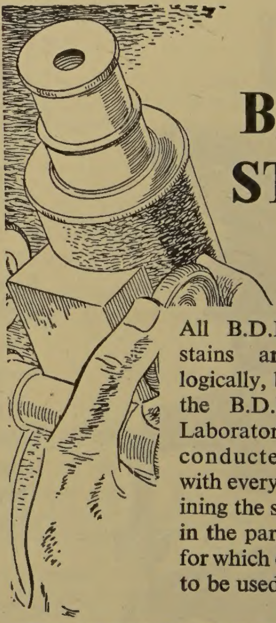

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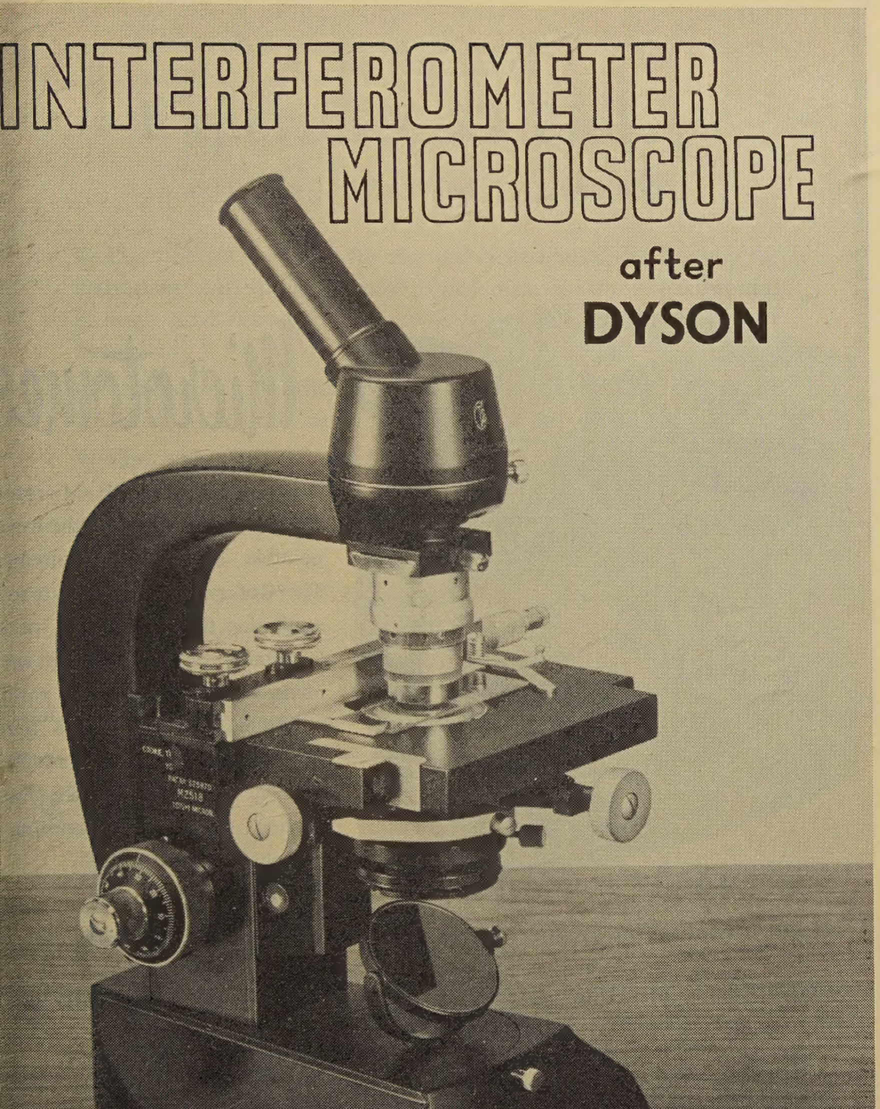
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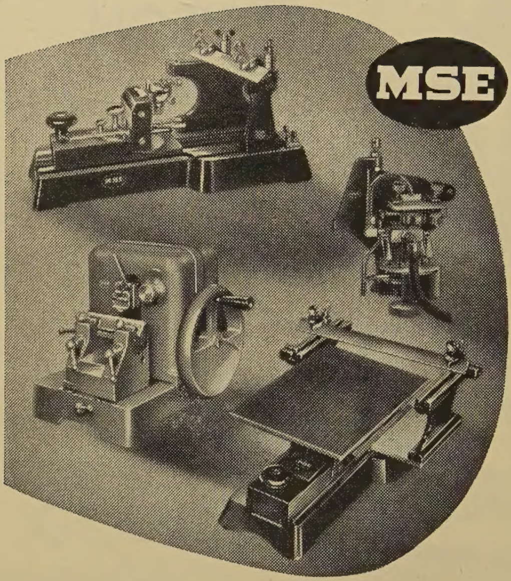

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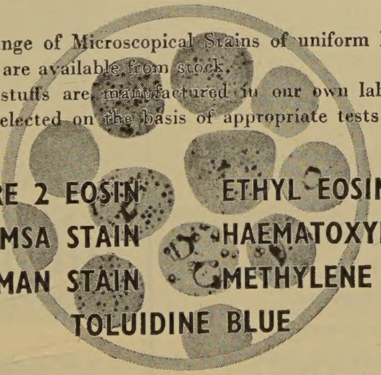

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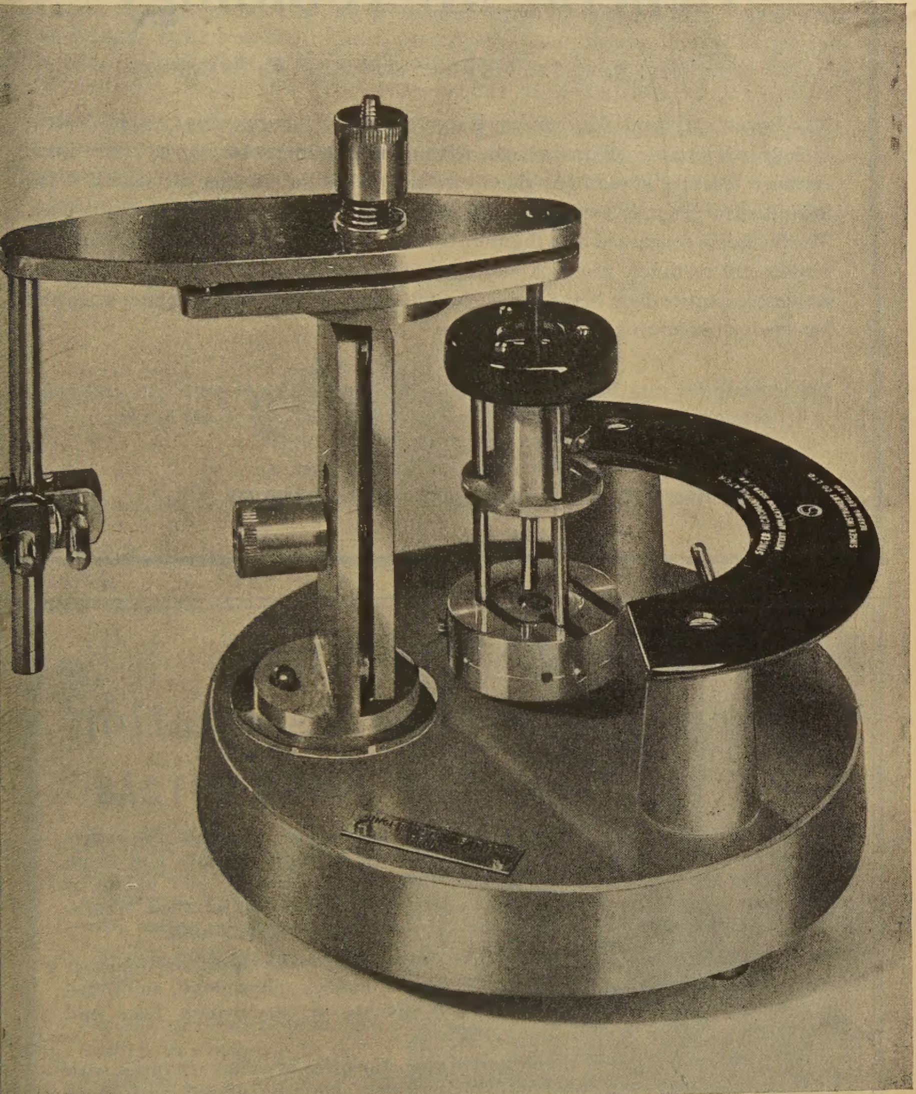

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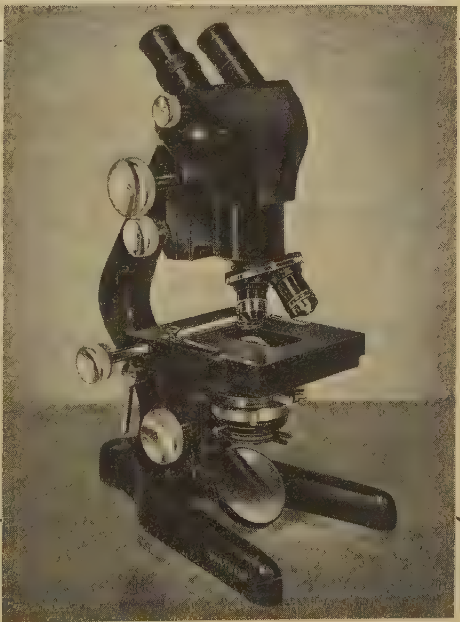

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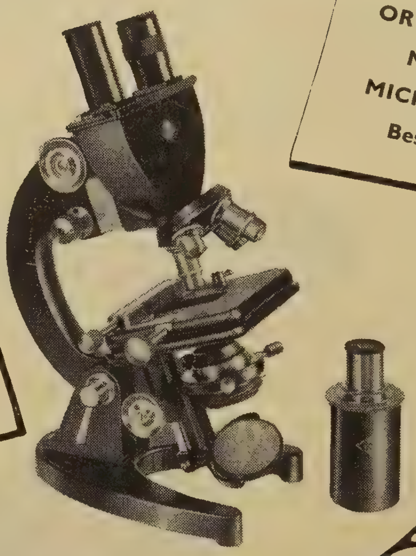

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Refractometry of Living Cells

Part I. Basic Principles

By R. BARER AND S. JOSEPH

(*From the Department of Human Anatomy, Oxford*)

With one plate (fig. 1)

SUMMARY

The principles underlying a new method of refractometry of living cells are dis-sed. The method was evolved from the chance observation that the amoebocytes he blood of the earthworm, examined in their own blood, appeared bright instead lark by positive phase-contrast microscopy. This was shown to be due to the pre-ce of dissolved haemoglobin which raised the refractive index of the medium above t of the cytoplasm. In order to determine the refractive index of the latter it was y necessary to dilute the blood until the cytoplasm became virtually invisible. n-pigmented proteins and other high molecular weight substances have now been stituted for haemoglobin.

The nature of the initial observations suggested that if the cell could be regarded to rst approximation as being composed entirely of proteins, the cytoplasmic protein centration could be equated to the protein concentration of the immersion medium ich made the cell appear with minimum contrast. This would only be true if equal centrations of different proteins in solution had the same refractive index. The ure of refractive index and its relationship to density are discussed and it is shown t for nearly all unconjugated proteins so far investigated the specific refrac-increments (i.e. the increase in refractive index per 1 per cent. increase in con-tration) have almost the same values (·00185±2 per cent.). The effects of many tors such as pH, salts, temperature, wavelength, concentration, and nature of the vent are discussed. Since living cells contain substances other than proteins the cific refraction increments of protein derivatives, lipides, carbohydrates, and salts considered and it is shown that the presence of moderate amounts of such sub-nces is unlikely to affect the refractive index of cells to any great extent. It is sug-ted that the mean specific refraction increment of protoplasm should be taken as 18 and that this value can be used in order to calculate the solid and water content protoplasm from values of refractive index.

CONTENTS

PAGE
MMARY 399
RODUCTION 400
MARKS ON THE THEORY OF REFRACTION 402
  Refractive index and density 403
  The concept of specific refraction increment 405
  The refraction increment of protein solutions 407
    Pigmented proteins 408
    Effect of pH 410
    Effect of salts 411

uarterly Journal of Microscopical Science, Vol. 95, part 4, pp. 399–423, Dec. 1954.]

Effect of temperature . . . .
Effect of wavelength . . . .
Effect of concentration . . . .
Effect of medium . . . .
THE REFRACTION INCREMENT OF CERTAIN CELLULAR CONSTITUENTS . . . .
Protein derivatives and constituents . . . .
Nucleic acids and nucleoproteins . . . .
Lipids and lipoproteins . . . .
Carbohydrates . . . .
Inorganic constituents . . . .
THE REFRACTION INCREMENT OF PROTOPLASM . . . .
REFERENCES . . . .

INTRODUCTION

VERY little work has been carried out in the past on the measurement
the refractive index of living cells. This is surprising in view of the num
ous investigations of other physical properties of protoplasm such as viscos
elasticity, density, absorption of light of different wavelengths, &c. Even
the case of the few values which have been obtained no attempt appears
have been made to interpret the results, and the latter were usually quoted
bare figures for the refractive index which seemed to have little meaning
themselves. With the advent of phase-contrast microscopy, interest in refr
tometry revived somewhat, as it was soon observed that the appearance
many objects varies greatly with the refractive index of the mounting mediu
Investigations on fixed and dehydrated tissue sections showed that the
fractive index of such materials was usually about 1·53–1·54 (Crossmon, 19
This value was determined by examining the specimen by phase-contr
microscopy, using mounting media of different refractive indices. When
refractive index of the medium matched that of the tissue, the latter beca
virtually invisible. This technique is essentially the well-known immers
method which has long been used in mineralogy and crystallography. It
not appear possible to use immersion methods for measurements on liv
as opposed to dehydrated cells, because most of the common immers
media were incompatible with viability. Some biologists apparently imagi
that the values obtained for tissue sections could be applied to living pro
plasm without realizing that the considerable hydration of the latter must
evitably lower its refractive index. This was perhaps another reason why
search for suitable immersion media was not pursued more energetically
aqueous media with a refractive index exceeding 1·50 are comparatively
common.

The development of interference microscopy, which in some cases can
used for the quantitative determination of optical path difference, at first si
seemed to offer a solution to the problem, but since the optical path differe
depends on the *product* of refractive index and thickness, the refractive in
itself cannot be measured without some method of determining the thickr
of living cells. This determination is subject to very considerable errors.

The method which we have developed depends on a purely chance obser

which not only indicated how the problem might be solved but suggested
imple practical interpretation. Both the method and the interpretation
y seem obvious now, but they were not so at the time.

The initial observation was made at the beginning of 1952 during an ex-
ination of various types of amoebocytes with the phase-contrast micro-
pe (Barer and Ross, 1952). The purpose of this investigation was in no
y concerned with the measurement of refractive index, but was an attempt
find suitable material for studying the action of ultra-violet light on living
ls. It was thought that the free-living amoebocytes in earthworm blood
ght provide a readily available source of suitable material. Blood was
erefore drawn from the region of the hearts by means of a fine pipette and
amined by phase-contrast microscopy. At first sight the appearance of
ny of the amoebocytes was disappointing as the contrast seemed to be un-
pectedly low. We then observed to our surprise that the cytoplasm of many
the cells appeared *bright* instead of dark when a positive phase-contrast ob-
tive was used (fig. 1). Under the conditions of the experiment the only
sonable explanation seemed to be that the refractive index of the cytoplasm
s less than that of the surrounding fluid. This was astonishing to us, as
was assumed at first that the refractive index of the latter was roughly the
me as that of water. On further consideration, however, it was realized that
like mammalian blood, earthworm blood contains *dissolved* haemoglobin,
d that if the latter were present in sufficient concentration it might con-
ivably raise the refractive index of the medium above that of the amoebocyte
toplasm. Simple experiments showed that this explanation was correct. On
nning in isotonic saline solution beneath the coverslip, the cytoplasm
adually faded, disappeared, and then became progressively darker, until
er several changes of saline solution it remained constant in appearance and
owed the dark contrast normally associated with other types of amoebocyte
en viewed by positive phase-contrast. It was therefore clear that this
ethod could be used to determine the refractive index of the cytoplasm of
ese cells; all one had to do in principle was to dilute the blood with a suit-
le saline solution until the cytoplasm, as far as one could judge, disappeared
phase-contrast microscopy. At the same time a possible interpretation of
e meaning of refractive index suggested itself. If the cell could be regarded
a first approximation as being composed entirely of protein, could it be
sumed that at the disappearance point the concentration of protein in the
mersion medium was equal to that within the cytoplasm? Further experi-
ents and a detailed search of the literature showed that this was in fact a
asonable assumption. Aqueous solutions of all proteins so far investigated
ve very nearly the same refractive indices for the same concentration.
oreover, other substances likely to be present in protoplasm, particularly
ides and carbohydrates, would for reasons discussed below not be ex-
cted greatly to affect this basic conclusion. In fact *the refractive index of a
ing cell can be taken as a measure of the total organic solids present.* The
idence for this statement will be discussed in detail later. It was also

apparent that if the concentration of solids could be determined, that water could be obtained by simple subtraction. In other words, a comparative simple measurement under the microscope could yield quantitative inform tion concerning two very important cellular constituents.

Having established the feasibility of the method, a search was carried out f more suitable immersion media than haemoglobin solutions. Althou haemoglobin is not difficult to prepare, and large quantities are readily ava able, its deep colour may be a disadvantage and, so far as is known, suf ciently pure material is not commercially obtainable. A colourless commerciall available protein was obviously preferable, and we were fortunate in findi at once a material which proved to be suitable. This was Armour's bovi plasma albumin, fraction V, which is sold in the form of a dry powde Crystalline bovine plasma albumin is also produced by the same firm, b does not show any special advantages and is much more expensive. Man other substances have been investigated as possible substitutes for plasm albumin, and although one or two appear to be promising for certain pu poses, none has been so universally successful. Even plasma albumin, how ever, suffers from certain disadvantages, which will be mentioned later, and is hoped that a more suitable substance will eventually be discovered.

As the technique and interpretation of the results are quite new, we ha felt that a detailed consideration of the practical methods and the vario difficulties associated with them, together with a discussion of the underlyi fundamental principles, would be useful to workers in many fields in whi the method is applicable. This paper is therefore concerned with gener principles rather than with detailed results. The latter will be described els where in relation to the various problems investigated. Some indication the results will, however, be given in order to compare them with the figur for solid and water concentration obtained by other methods (see also Bare Ross, and Tkaczyk, 1953).

The length of this communication makes it necessary to publish it separate parts. The first part is concerned with fundamental principle Later parts will deal with the properties of the immersion medium, technic methods and precautions, interpretation of results in terms of solid and wat content of cells, estimation of errors, and comparison with other methods.

REMARKS ON THE THEORY OF REFRACTION

Many aspects of the theory of refraction of electromagnetic waves are w understood, and the subject has been investigated mathematically both

---

FIG. 1 (plate). Positive phase-contrast photographs of a living earthworm amoebocyte an isotonic solution containing 16 per cent. bovine plasma albumin. This cell was origina observed in the animal's own blood, and appeared faintly reversed in contrast. The blood w then replaced by the protein solution and the cytoplasm has become strongly reversed. T reversal is seen in both photomicrographs. These pictures are from a series showing the c in active motion and apparently in good health. Note how the disposition of the inclusio bodies changes during movement. The fibrous structures around the cell are spermatoz with which the preparation became contaminated.

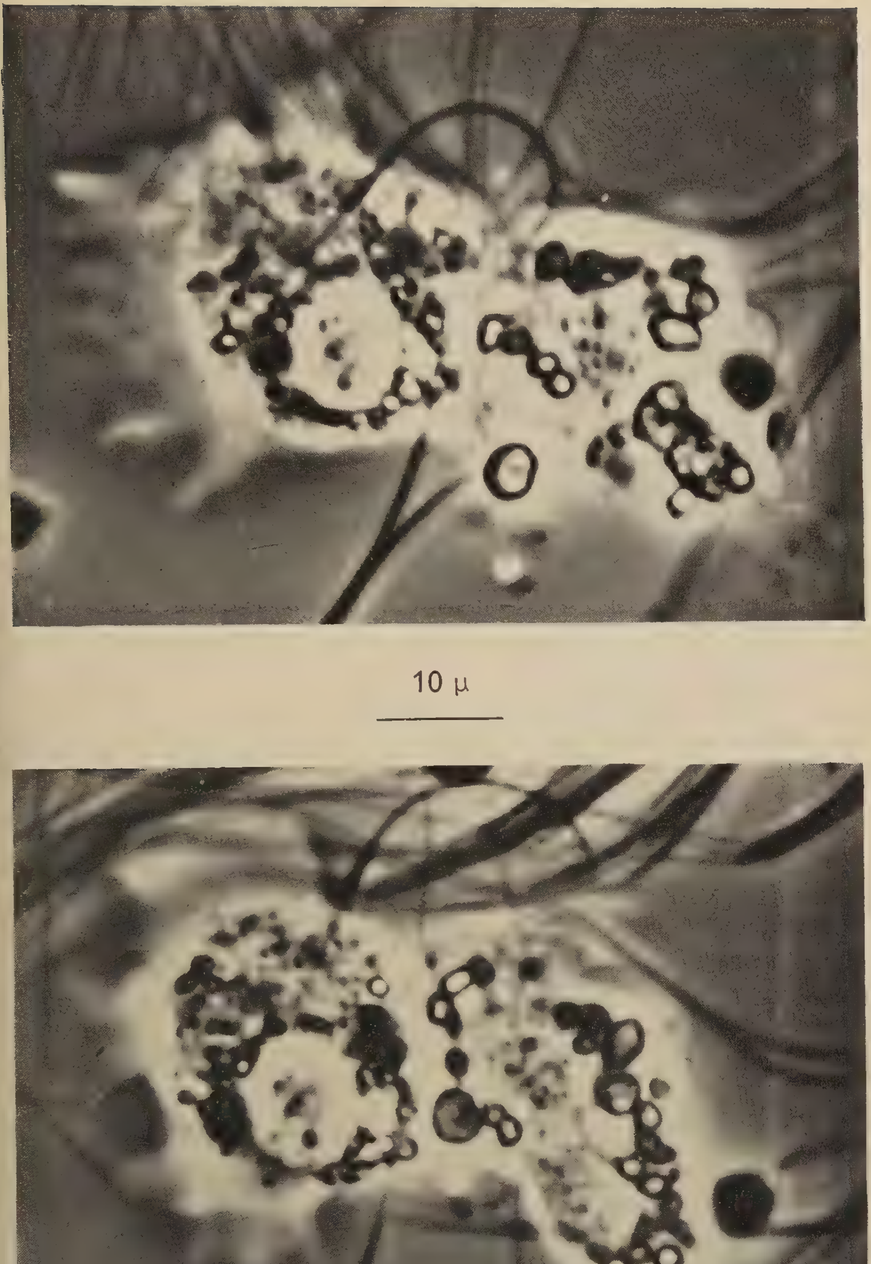

FIG. 1

R. BARER *and* S. JOSEPH

ssical Electromagnetic Theory and by Quantum Theory. The results are sentially identical. Unfortunately the theory is rather complex and it is fficult to give a satisfactory explanation based on simple physical concepts ne. The remarks which follow must not be regarded as a rigorous treatment the subject, but may be helpful in indicating in an approximate manner the rt of factors which are responsible for the refractile properties of different bstances.

According to classical theory an electromagnetic wave falling on an atom molecule may set the whole molecule and its electron shells into vibration. the case of ultra-violet and visible light it is mainly the outer electrons hich are made to vibrate. These do so with the same frequency as the cident wave, but in general with a different phase. The vibrating electrons nit secondary light waves. The secondary waves interfere destructively with ch other in a lateral direction, but in the original forward direction they not disappear but combine with the primary wave, altering its phase and this way changing the wave velocity. The ratio of the velocity of light *in cuo* to its velocity through a transparent substance is defined as the refracve index n. The phase of the forced vibrations which the electrons undergo epends on the frequency of the incident light wave, so that the refractive dex will also depend on this frequency and hence on the wavelength.

Refractive index and density

We can see in a general way that the refractive index of a substance is reted to its density. A light wave passing through an empty space will enunter no atoms, and it will proceed with unchanged velocity. If the same ace is now partially filled with atoms or molecules, the incident wave will teract with these as described above, so that its velocity will be reduced. t is reasonable to assume that since the reduction in velocity is the result of e interaction between the light and atoms in its path, the refractive index ill depend on the number of the latter per unit length, or in other words on e density. The difference in optical path due to the presence of matter deends on $n-1$, so that the quantity $\frac{n-1}{d}$, where d is the density, might be exected to be approximately constant. This was in fact suggested and verified xperimentally by Gladstone and Dale (1858, 1863). For a given wavelength ey found that $\frac{n-1}{d}$ was almost independent of temperature for many quids. This empirical relationship was given a sounder theoretical basis by I. A. Lorentz (1880) and L. V. Lorenz (1880), who calculated independently rom the electromagnetic theory of light that the quantity $\frac{n^2-1}{n^2+2}\cdot\frac{1}{d}$ should be constant. This expression can be written in the form $\frac{n-1}{d}\left(\frac{n+1}{n^2+2}\right)$, which is simply the Gladstone–Dale expression multiplied by the term in bracke The latter only varies slowly with n. Thus it falls from ·62 when $n = 1{\cdot}3$, to · when $n = 1{\cdot}6$, a range of n covering most common liquids. Hence t Lorentz–Lorenz relationship approximates to the simpler Gladstone–Da formula. In fact both formulae express the behaviour of many substanc quite well and the quantity R defined as

$$R = (n-1)\,.\,\frac{M}{d}$$

or

$$R = \frac{n^2-1}{n^2+2}\,.\,\frac{M}{d},$$

which is known as the molecular refractivity, is a characteristic property of given molecule (M = molecular weight). The Lorentz–Lorenz formula usually preferred to the Gladstone–Dale one because it is assumed to have sounder theoretical foundation, but it must be remembered that both a approximations, and, indeed, in a few cases it has been shown that the Gla stone–Dale expression fits the experimental facts rather better. One mu beware therefore of ascribing a greater validity to the Lorentz–Lorenz r lationship than it really possesses. The assumptions on which it rests ha been critically examined by Böttcher (1952), Oster (1949), and others. Anoth relationship based on theoretical considerations which attempt to elimina some of the difficulties inherent in the Lorentz–Lorenz theory is that Onsager (1936). His formula is

$$\frac{(n^2-1)(2n^2+1)}{9n^2}\,.\,\frac{1}{d} = \text{const.}$$

This can be written in the form

$$\frac{n-1}{d}\left[\frac{(n+1)(2n^2+1)}{9n^2}\right] = \text{const.}$$

This more complicated formula again approximates to the simple Gladstone Dale relationship, and, indeed, the quantity in square brackets changes extreme slowly with n. Thus it varies only from ·662 to ·694 as n varies from $1{\cdot}3$ to 1· Onsager's formula can thus be regarded as the theoretical justification of th Gladstone–Dale relationship, and it has been shown to fit many experiment facts very well (Oster, 1949).

One of the assumptions on which the Lorentz–Lorenz relationship is base is interesting in that it gives us a further physical insight into the nature refraction. This assumption is that the molecules of a substance are conduc ing spheres. According to theory it turns out that if n is measured at very lon (strictly infinite) wavelengths, the molecular refractivity is nothing more tha the actual volume of these spherical molecules in one mol of the substanc This is, of course, only a comparatively crude approximation for real sub nces, but it is nevertheless a useful concept. It suggests, for example, that if imagine a vessel containing given numbers of atoms of different kinds the ractivity of the contents of the vessel will be independent of the way in ich these atoms are combined, assuming that the volume they occupy will t be very much changed in different combinations. Also, if we changed one t of atom for another having about the same volume, there would again little change in refractivity. If only a small proportion of the atoms present re changed for others of a very different size, the refractivity would again t be greatly affected. These predictions are with certain important excep- ns roughly borne out in practice. It has been shown that the molecular raction is an additive property. By measuring the refractive indices of a ge number of substances it has been possible to deduce atomic refractivi- s for different types of atoms and in this way to predict the molecular re- ctivity of many substances. In some cases the agreement between theory d experiment is remarkably good. Experience has shown, however, that omic refractivities do in some cases depend on the way in which an atom bound to its neighbours. Thus the atomic refractivity of oxygen in a car- nyl group is nearly 50 per cent. greater than that of oxygen in a hydroxyl oup. The presence of double and treble bonds raises the molecular refrac- ity, and the influence of conjugated double bonds is particularly strong in is respect. Many substances which are visibly pigmented contain groups this type, and show so-called 'anomalous' dispersion or refraction in the gion of an absorption band (see Wood, 1934; Ditchburn, 1952). In regions mote from absorption bands the refractive index changes comparatively owly with wavelength.

These principles lead to certain important conclusions when applied to ganic substances, especially those of high molecular weight. Since most irly large organic molecules are composed mainly of carbon, hydrogen, nitro- en, and oxygen, one might not expect very great differences in refractile operties provided that not many unsaturated groups are present. This is ughly true, and gross departures only occur when atoms such as sulphur iodine, for example, are introduced into a molecule. In the case of ex- emely large molecules such as the proteins even quite big differences in the mino-acid composition would be unlikely to affect the refractive index gnificantly. The unpigmented proteins show no specific absorption bands the visible region, so that anomalous refraction is only to be expected in the ltra-violet (at about 280 mμ) where the absorption due to the aromatic mino acids becomes manifest. In the visible region, therefore, all proteins ould be expected to have roughly the same refractive indexes.

The concept of specific refraction increment

If we consider the Gladstone–Dale relationship in the form $\frac{n-1}{d}$ = con- tant, it is possible to derive a formula connecting refractive index and con- entration. If the density of a solution can be taken as varying linearly with the concentration of dissolved substance, the Gladstone–Dale formula can written

$$n = K+\alpha C,$$

where n is the refractive index of the solution whose concentration is C, an K and α are constants. By writing $C = 0$, we see that $K = n_m$, the refracti index of the pure solvent. Hence

$$n-n_m = \alpha C.$$

This formula has been found to hold experimentally for a large number different substances, *provided that the concentration C is measured in terms weight per unit volume*, i.e. grams of solute per 100 ml. of solution. α th becomes the increase in refractive index of the solution for every 1 per cen increase in concentration, and is known as the *specific refraction increment.*

The question sometimes arises whether, given the value of α, we can ca culate by extrapolation the refractive index of the pure dry solute. It is im portant to observe that this cannot be done simply by substituting $C = 100$ the formula, as might appear at first sight. C is the number of grams dissolv in 100 ml. of solution and 1 gram of a substance may occupy more or less tha 1 c.c., depending on its density. In the case of proteins in solution, for exampl the density can be taken as approximately 1·33, so that 133 grams of prote would occupy 100 ml. of 'solid solution'. On this basis the refractive index dried protein should be given by $n = n_m+133\alpha$. Taking n_m as the refracti index of water, namely, 1·333, and α as ·00185 (in water), the refractive ind of pure protein should be approximately 1·58. This is in quite good agre ment with many of the published figures for the refractive indices of prote crystals, though exact comparison is difficult because such crystals are oft strongly birefringent and the refractive index varies with the axis of measur ment. The refractive index of pure proteins can also be calculated by extr polation, by using the Lorentz–Lorenz relationship. This has been done Putzuys and Brosteaux (1936), who found that the refractive index of mo proteins was approximately 1·60. This is only a little higher than the valu given by the Gladstone–Dale formula and it is not in fact in any better agre ment with the rather scanty published results. It is rather remarkable th both formulae give figures which are higher than the refractive indexes dried protein fibres and gels, which are generally stated to have a refracti index of about 1·53 to 1·54. The exact reason for this discrepancy is n known. It may be that the so-called 'dry' gels and fibres still contain a co siderable amount of water, though one might expect the same to hold protein crystals. Another possible factor is that we have considered t density of proteins to be the same in the dissolved state as in the solid stat This may not be the case, and the density may differ in dried gels or crystal These are difficult theoretical problems, but it is clear that values for refra tive indices of solids obtained by extrapolation must be regarded with som caution. The same applies to the reverse procedure of attempting to derive t lue of α from a knowledge of the refractive index of a solid. This is illus-ated in a note published by Davies and Wilkins (1952). The value of α easured for dilute solutions of tobacco mosaic virus is ·0017, whereas that lculated from the refractive index of the dry solid (1·534) is ·0015. The discre-ancy in the case of inorganic salts is even greater. Thus for aqueous solutions sodium chloride α = ·00163, whereas the value calculated from the proper-es of the solid is only ·00097. The corresponding figures for calcium chloride e ·0021 and ·00075. In view of these facts it is evident that the calculation α from the properties of the solid is highly dangerous, and it is essential to ly only on results which have been determined experimentally for solutions.

The refraction increment of protein solutions

Refractometry was suggested as a method for estimating protein concentra-on as early as 1903 by Reiss. He was particularly concerned with the estima-on of blood serum proteins, and obtained values of α of ·00183 for serum lbumin and ·00224 and ·00230 for two different pseudoglobulin fractions. he following decade saw a great expansion of work on the subject, due to the nfluence of T. B. Robertson, who investigated the refractile properties of many roteins and used refractometry for measuring the concentration of various rotein constituents of the blood. A summary of this work and references to he considerable early literature on the problem will be found in Robertson's ook on the physical chemistry of proteins (1918). Robertson's work was etailed and careful and he undoubtedly laid the foundations for future exact ork in this field. He was, however, hampered by the comparative crudity f methods for separating and purifying proteins which were available in his ay, so that many of his samples were probably not very pure by modern tandards. Nevertheless, the values he obtained for the refraction increments f several proteins are quite close to those obtained by modern workers. In ddition he investigated many basic problems such as the effects of concen-ration, temperature, and pH.

Modern work on the subject may be said to have been instituted by Adair nd Robinson in 1930. They introduced two main advances, namely, the use f more highly purified samples and the application of the Donnan equilibrium heory to calculate the salt concentration in solutions of proteins purified by ialysis. This is particularly important in the case of those proteins which are nly soluble in the presence of salts, for the Donnan effect results in an nequal distribution of ions between the protein and its solvent. In such cases dair and his collaborators have distinguished between α, the refraction ncrement in water, and α', the refraction increment in the presence of a onnan distribution of ions. In general, α and α' differ by only a very small mount. In their work on horse serum albumin, Adair and Robinson used aterial which had been recrystallized several times and dialysed for varying eriods. They found that between 14·7° and 20·1° C. and over the concentra-ion range 2·9–8·47 per cent. α was essentially constant, the extreme values eing ·001816 and ·001852, with a mean of ·0018295.

In the next twenty years steady improvements were made in the separatio and purification of proteins, particularly by means of ultracentrifugation ar electrophoresis. A knowledge of α is important for quantitative work li these methods, as it is also for the determination of molecular weights b light-scattering measurements in which the value of α^2 is used. These metho have thus stimulated interest in work on refractometry of proteins and allie substances.

It will be convenient at this stage to tabulate some of the main resul obtained by different workers and to discuss the influence of various factor In a few cases details such as pH, concentration range, temperature, ar wavelength used are given, but unfortunately such facts are not alwa stated in published work, and slight discrepancies in results may be due diverse experimental conditions. Another important cause of differenc is that there is considerable variation in the method of estimating the prote concentration. Some authors have used dry weight measurements, others hav used weight of crystalline protein, and many have estimated the nitrogen concentration by Kjeldahl's method. In the latter, in order to convert the nitrogen content to protein concentration, it is necessary to use a conversion facto which varies slightly for different proteins and whose value is not very precisely known even for a given protein. When, however, due allowance made for these various factors, it is found that the results for a given protein obtained by different authors show very good agreement. For exampl some of the values obtained by Halwer and others (1951) differ fro those of Perlmann and Longsworth (1949) by nearly 2 per cent., even whe differences of temperature and wavelength are taken into account. The forme workers estimated concentration by drying samples to a constant weigh whereas the latter used Kjeldahl nitrogen determinations.

If we ignore the figure for total human serum, which contains substance besides proteins, the lowest value for α in table 1 is ·00181. The highest valu measured with green or yellow light and at approximately room temperatur is ·00188. Taking a mean of ·001845, the extremes differ from this b only ±2 per cent. Considering the diversity of materials and methods use by various workers this degree of agreement is remarkable, and one migh almost say exceptional, in biological work. Table 1, however, only refers t unconjugated soluble proteins.

Pigmented proteins

A number of figures for the refraction increments of haemocyanins an haemoglobins are available. These have been collected in table 2. It shoul be noted that the values given by Redfield (1934) are for haemocyanins dis solved in water, whereas the figures of Adair and his collaborators refer t haemocyanins dialysed against phosphate buffers at pH 7·5. Adair's result therefore are for α' rather than for α. The same applies to his values fo haemoglobins, with the exception of Stoddard and Adair's figure for huma haemoglobin. The value obtained by Howard (1920) for haemoglobin i nsiderably less than that found by Stoddard and Adair (1923). This may rhaps be accounted for by the fact that the latter workers based their concen- ation measurements on weight of oven-dried protein, whereas Howard ed the weight of crystals, which contain water. On the whole the values for

TABLE I

Specific refraction increment (α) *of unconjugated proteins*

Protein	*C*	*pH*	*T*	λ	α	*Reference*
ovine serum albumin	3·7–10·1	5·05	·5°	5780	·001901	a
,, ,,	3·7–10·1	5·05	20°	5780	·00187	a
,, ,,		5·2	25°	4360	·001924	b
,, ,,		5·2	25°	5460	·001854	b
orse serum albumin	2·9–8·5		14–20°	white	·001830	c
,, ,,		4·8		4360	·001912	b
,, ,,		4·8		5460	·001844	b
uman total serum				white	·00179	d
,, ,, albumin				white	·00181	d
,, crystalline albumin				white	·00181	d
,, serum albumin	1·78–7·7	4·85	·5°	5780	·001887	a
,, ,,	1·78–7·7	4·85	20°	5780	·001862	a
,, pooled plasma	3–6		25°	5893	·001833	e
,, crystalline albumin	5·1–14·6	4·4–7·7	25°	5893	·001860	e
gg albumin					·001813	f
,,	·74–6·6	4·5–5·8			·001854	g
,, (denatured)	·65–6·6	6·9–7·4			·001844	g
,,	1·6–6·45	4·95	·5°	5780	·001876	a
,,		4·8	25°	4360	·001883	b
,,		4·8	25°	5460	·001820	b
uman γ globulin			25°	5893	·00188	e
,, ,,			·5°	5780	·001875	a
orse serum globulin	3·3–17			white	·00186	c
uman α_2 globulin				5893	·00183	e
,, β_1 globulin				5893	·00185	e
,, euglobulin					·00183	d
,, pseudoglobulin					·00181	d
actoglobulin	2			4360	·001892	h
,,	2			5460	·001818	h
lactoglobulin		5·2		4360	·001890	b
,,				5460	·001822	b
uman fibrinogen			25°	5893	·00188	e

The concentration range *C* is given in terms of grams of protein per 100 ml. of solution. emperature *T* in degrees centigrade. Wavelength λ in Ångström units.

Key to references: a, Perlmann and Longsworth, 1948; b, Halwer and others, 1951; Adair and Robinson, 1930; d, Adair, 1952; e, Armstrong and others, 1947; f, Taylor and hers, 1932; g, Barker, 1934; h, Pedersen, 1936.

proteins containing pigment groups are higher than those for unpigment proteins as is to be expected on theoretical grounds. Redfield's value of ·o for the specific refraction increment of Homarus haemocyanin is probab the highest fairly reliable figure reported for aqueous protein solutions. It c be stated with reasonable certainty, therefore, that the refraction incremen of nearly all known proteins will lie within the range of ·00170–·00200.

TABLE 2

Specific refraction increment of pigmented proteins

Protein	*C*	*pH*	*T*	*γ*	*α*	*Referenc*
Haemoglobin, Ox, CO		7·4		white	·001930	i
,, ,, ,,		5·6		white	·001935	j
,, ,, ,,		7·7		white	·001988	j
,, sheep, CO				white	·001945	k
,, human	·04–8·17			white	·001942	l
Haemocyanin *Helix*		7·5		white	·00179	m
,, *Octopus*		7·5		white	·00184	m
,, ,,	1·1				·00197	n
,, *Carcinus*		7·5		white	·00187	m
,, *Homarus*	11·85		20°		·002	o
,, *Limulus*	4·9–7·3		15–23°		·00198	o
,, *Busycon*	1–11		20–24°		·00197	o

Headings as in table 1.

Key to references. i, Adair and others, 1946; j, Roche and others, 1932; k, Adair and Ada 1934; l, Stoddard and Adair, 1923; m, Roche and others, 1935; n, Quagliariello, 1920; Redfield, 1934.

Effect of pH

Except at their isoelectric points proteins normally exist as salts. Since such cases salt formation consists in the replacement of a relatively small nur ber of hydrogen or hydroxyl groups by, for example, sodium or chloride ion it would seem inherently unlikely that a change of pH would have very mu effect on the refractive index of large molecules such as proteins. This h been borne out experimentally. Adair and Robinson (1930) prepared sol tions of sodium albuminate by dialysing against buffers at pH 7·4. Und these conditions there is an unequal distribution of ions due to the Donna effect, so that α′ is measured. The mean value found for α′ for blood album from various sources was ·00177. The value of α calculated according to th Donnan equations was ·00180, in reasonably good agreement with the experi mental result of ·00183 for crystalline isoelectric albumin. Adair and Robinso point out that the difference between α and α′ depends on the concentr tions of hydrogen ions, salts, and the protein. They quote one instance which the difference between α and α′ was negligible. In the case of hor serum globulin, which is insoluble in pure water, α′ was determined at bo pH 5·35 and pH 7·4. The values obtained were ·001815 and ·00181 respe tively. α was calculated to be ·001857 and ·00187 respectively, so that th influence of pH was very small.

The effect of pH was also investigated by Perlmann and Longsworth (1948) two different ways. In the first method they determined the refraction in- ement of salt-free isoelectric proteins to which small amounts of sodium droxide had been added. For egg albumin the refraction increment in- eased from ·001877 at pH 4·95 to ·001909 at pH 10·5. For bovine serum umin the value increased from ·001902 at pH 5·02 to ·001979 at pH 10·72. ll measurements were carried out at ·5° C.) They concluded that the specific fraction increment α_H at any pH was related to the specific refraction incre- ent at the isoelectric point α, by the formula

$$\alpha_H = \alpha(1+ae),$$

ere e is the net charge on the protein and a is a constant whose value was termined experimentally to be approximately 45. The second method used as to change the pH by dialysis against buffers, taking into account the onnan equilibrium. When sodium phosphate buffers were used, the results tained agreed very well with those for the isoelectric solutions treated with ustic soda. With diethyl barbiturate buffers, on the other hand, the results ere slightly higher, a tentative explanation being that the diethyl barbiturate n was bound by the protein.

Although the investigations of Adair and Robinson and of Perlmann and ongsworth on the effect of pH are the most detailed and reliable so far ailable, it should be mentioned that other workers have frequently noted passing that the effect of pH was either small or negligible (Robertson, 18; Barker, 1934; Armstrong and others, 1947).

ffect of salts

Perlmann and Longsworth carried out measurements on egg albumin, ovine serum albumin, and human serum albumin, equilibrated by dialysis ainst various strengths of sodium chloride solutions. According to their sults there is a slight rise in α for bovine and human serum albumin as the lt concentration is raised. The effects are small, and in the case of bovine rum albumin the maximum increase obtained was about 2 per cent. Egg bumin, on the other hand, appeared to be unaffected by salt concentration. alwer and others were unable to confirm these results and found that α was ot affected by salt concentration.

ffect of temperature

Most workers were unable to observe any significant change of α with tem- erature, possibly because they did not work over a sufficient range. Perlmann nd Longsworth have published curves showing the variation of α with tem- erature between 0·5° and 25° C. for bovine and human serum albumin, gg albumin, and β lactoglobulin. In these cases there was a slight increase mounting to about 0·5 per cent. as the temperature fell from 25° to 5° C., ith a slightly steeper rise towards 0°. In the case of human albumin α only

rose from ·001854 at 25° to ·001887 at ·5° C. Human γ globulin showed
negligible variation of α with temperature. Kenchington (1954) has also foun
that the value of α for gelatin is virtually independent of temperature.

Effect of wavelength

Most measurements of α are carried out with either green or yellow lig
from mercury or sodium lamps with appropriate filters. Some worker
notably Adair, have used white light, in which case the wavelength of measur
ment probably corresponds to the maximum spectral sensitivity of the eye

TABLE 3

Variation of specific refraction increments (α) *with wavelength*

λ (*m*μ)	*Serum albumin*	*Serum globulin*	*Lactoglobulin*
366	·00198	·00202	·001963
436	·00190	·00195	·001892
546	·00185	·00187	·001818
579	·00183	·00186	·001810
589	·00183	·00186	
656	·00179	·00182	

The figures for serum albumin and serum globulin are by Pedersen and Andersson, quot
by McFarlane (1935). The figures for lactoglobulin are by Pedersen (1936).

the green region. The refractive indexes of proteins rise considerably as th
wavelength is shortened, and even over the visible spectrum there is an appr
ciable change in α. The widest range of wavelength so far used for the dete
mination of α is from 366 to 656 mμ, as employed by Pedersen and Anderss
(quoted by McFarlane, 1935). Their results are given in table 3. Perlman
and Longsworth also carried out measurements over a narrower spectr
range and found that their results as well as those of Pedersen and Anderss
could be expressed by a formula of the type

$$\alpha_\lambda = \alpha_0\left(A + \frac{B}{\lambda^2}\right),$$

which is similar to the well-known Cauchy dispersion formula (see Woo
1934; also Putzuys and Brosteaux, 1936). Since the effect of wavelength on
is quite considerable, it is generally advisable to work in the green or yello
regions of the spectrum or with white light sources in which the blue com
ponents are naturally weak or have been filtered out. The combination of th
spectral sensitivity of the eye and the spectral emission of ordinary tungste
filament lamps ensures that for visual work with such sources the intensity
the blue rays is negligible as compared with that of the green and yellow ray

No detailed work appears to have been carried out on the variation of α wit
wavelength in the case of pigmented proteins such as haemoglobin and haem
cyanin. Since it is known that anomalous dispersion occurs in the region
absorption bands, one would expect α to show variations in such regions. Th
dispersion of refractive index of haemocyanin was in fact investigated b

tzuys and Brosteaux (1936), but no anomalies were observed. This is not ogether surprising, because in order to detect strong anomalous dispersion e absorption band should be very intense (i.e. the molecular extinction efficient should be high) and narrow. Haemocyanin has a rather broad sorption maximum in the yellow region, so that sharp changes of refractive dex would not be expected. Perhaps the most important factor, however, is at the chromophore group that is conjugated to the protein is small as mpared with the latter, so that the molecular extinction coefficient can never come very great. Possibly if the work of Putzuys and Brosteaux on haemo-anin were repeated with narrower wavelength intervals and perhaps a more nsitive method of measuring refractive index, such as an interferometer, ght anomalous dispersion might be detected. The same arguments apply the case of haemoglobin. The two visible bands in the yellow and green gions of the spectrum are far too weak for any but very slight anomalies of fractive index or refraction increment to be expected. The haem pigments ve, on the other hand, a very intense Soret band at about 415 to 420 mμ. he effective molecular extinction is of course greatly reduced by conjugation ith protein, but even so one might expect to find some anomalies of refrac-on. So far as is known no measurements of refractive index of haemoglobin ave been carried out in this region, but indirect evidence for the existence of omalous dispersion has been obtained (Barer, 1952 and unpublished obser-ations). As already noted, the specific refraction increments of pigmented roteins tend to be distinctly higher than those of unpigmented ones. In the se of the latter, anomalous dispersion would only be expected to occur in e ultra-violet at a wavelength of about 280 mμ. No figures are available for e refractive indexes of proteins in this region of the spectrum, but the work f Pedersen and Andersson already quoted shows a fairly considerable rise α even at 366 mμ.

ffect of concentration

Most work on specific refraction increment has been carried out on protein olutions of low concentration. In view of the fact that high concentrations of roteins may be encountered in living cells, it is especially important in relation o the present work to find out over what range of concentration of protein α emains constant. Nearly all the earlier workers stated that the refractive ndex of a protein solution varied linearly with concentration over the range tudied, though full details were rarely given. Adair and Robinson (1930) ound no significant change in α for horse serum albumin betwen 2·9 and ·5 per cent. Perlmann and Longsworth (1949) found only small and irregular ariations for bovine plasma albumin between 3·8 and 10·1 per cent. Arm-trong and others obtained very constant results for crystallized human lbumin between 5·1 and 14·6 per cent. The highest concentration range so ar investigated appears to be up to 17 per cent., in the case of haemoglobin Howard 1920). Stoddard and Adair (1923) also found that α was constant for uman haemoglobin up to 8 per cent.

Since in our work we have frequently used protein concentrations exceedin 30 per cent. it was felt necessary to investigate the variation of α with co centration over a much wider range than hitherto. The problem is not simple as one might at first suppose. One could in principle measure the r fractive index of a concentrated protein solution and then determine its co centration by drying a known volume or by nitrogen estimation on a know volume. Unfortunately, with increasing concentrations, particularly tho exceeding 30 per cent. or so, the bovine serum albumin preparations becom increasingly viscous and difficult to handle. For accuracy, therefore, rath large volumes would be required, necessitating the use of several grams protein for each reading. Since commercial bovine plasma albumin is mode ately expensive, the cost of such an investigation becomes an importa factor. Another obvious approach would be to weigh out a definite amoun of dried protein and make it up to a given concentration by addition of wate This, however, is quite impracticable because even with moderately concen trated solutions some of the protein, despite all reasonable precaution becomes caked into a hard mass which tends to adhere to the container o stirring rod, making it virtually impossible to remove all the protein from th latter and to measure the volume of the solution accurately. For these reason an alternative method of estimating protein concentration was sought. All pro teins containing the aromatic amino acids tyrosine, tryptophane, and pheny alanine show characteristic absorption bands in the ultra-violet at abou 280 mμ (Beaven and Holiday, 1952). For a given protein the extinctio coefficient for the peak in this region can be measured and hence the con centration of protein present in a given solution can be determined. Th method has the advantage of requiring very little protein. In the case of bovin plasma albumin a 1 cm. thickness of a ·2 per cent. solution gives an optic density of approximately 1·3. If the Beer–Lambert laws concerning the pro portionality between concentration of solute, length of optical path, an optical density were obeyed, there would be no difficulty in diluting the un known sample by a known amount, measuring its optical density for a give thickness, and thus deducing the concentration. It is well known that con centrated solutions of proteins show slight opalescence and possibly othe effects, such as polymerization or association, which result in some departur from these laws. In order to avoid this, therefore, our solutions were dilute accurately by varying amounts in order to obtain approximately the *same* fina optical density at 278 mμ. The final concentration was chosen so as to mak the optical density about 1·0 for a 1 cm. path-length. This corresponds t a concentration of about ·2 per cent. At this dilution the turbidity of the solu tion is negligible and the Beer–Lambert laws can be assumed to hold for moderate range of concentrations around this value.

The experimental procedure was as follows. Concentrated solutions o bovine plasma albumin were made in distilled water (the absorption of whic was carefully checked at 278 mμ and found to be negligible). The solution were allowed to stand for at least several hours, and preferably overnight, an re stirred carefully at intervals in order to ensure homogeneity. Several nples were taken from different regions of each solution and their refractive lices measured. These precautions were essential in order to obtain content results. In the early stages of this work the results were rather variable cause it was not realized that 'stratification' occurs in concentrated protein utions, so that there is a gradient of concentration from the surface to the ttom of the containing vessel. Refractive indices were measured on a Bellingm and Stanley Abbe-type refractometer at 20° C., with monochromatic low mercury light (λ 579 mμ). Readings were also taken with a hand gar refractometer in order to compare the accuracy of the two instruments. ne protein concentration was calculated roughly from the formula $= \frac{n-1{\cdot}3330}{\alpha}$, taking ·00185 for α. A small volume of the solution was easured in an accurately graduated volumetric pipette and diluted with tilled water to a final concentration of approximately ·2 per cent. in a 250 ml. lumetric flask. Great care is necessary in this part of the procedure, particuly when dealing with concentrated solutions. The high viscosity of the ter makes it extremely difficult to suck them up into a narrow calibre pette. It is important not to overshoot the mark, for if the viscous solution deposited on the walls of the pipette it will take a very long time to fall to e desired level. All solution on the outside of the lower end of the pipette ıst be carefully removed by repeated wiping with plugs of cotton wool aked in distilled water. Finally, one of the most potent sources of error s found to be the incomplete emptying of the pipette. Ordinary washing rough with a few changes of water is quite inadequate as the film of viscous otein adheres firmly to the walls, and between 50 and 100 rinses were cessary before the concentration reached a final stable value. The diluted ution was mixed thoroughly and several samples were selected for measur- the light absorption at 278 mμ. This wavelength was chosen because preliminary measurement of the absorption spectrum of bovine plasma umin, fraction V, showed that it was the position of the absorption maxiım. Measurements of absorption were carried out with a special Hilger vispek ultra-violet spectro-photometer with a non-standard extra-large artz prism. The slit width used corresponded to a band width of about mμ. Silica absorption cells 1 cm. thick were used.

If the measured optical density of a diluted solution is d and the number of nes it has been diluted as compared with the original concentration is s, then concentration of the original solution is proportional to $s.d$. (It should be ted that under the conditions of the experiment this is true whether or not Beer–Lambert laws hold.) If this quantity is plotted against the refractive dex of the concentrated solution, the variation of refraction increment with ncentration is found. In order to convert $s.d$ to actual concentration, a owledge of $E_{1\,\mathrm{cm.}}^{1\%}$ (i.e. the optical density of a 1 per cent. solution in a cm. path) is required. The main purpose of this investigation was to deterne the slope of the refractive index / concentration curve and not to measure the absolute value of α accurately. $E_{1\,\text{cm.}}^{1\%}$ was not determined e perimentally, therefore, but the value of 6·6 found by Cohn and others (194 was used. The concentration of protein was thus taken as $\frac{s.d}{6\cdot 6}$ and these valu are plotted against the refractive index n in fig. 2. It will be seen that wit the limits of experimental error the curve is a straight line. The accuracy least at high concentrations because of the various difficulties associated w the viscosity of such solutions. Nevertheless, the departure from linear

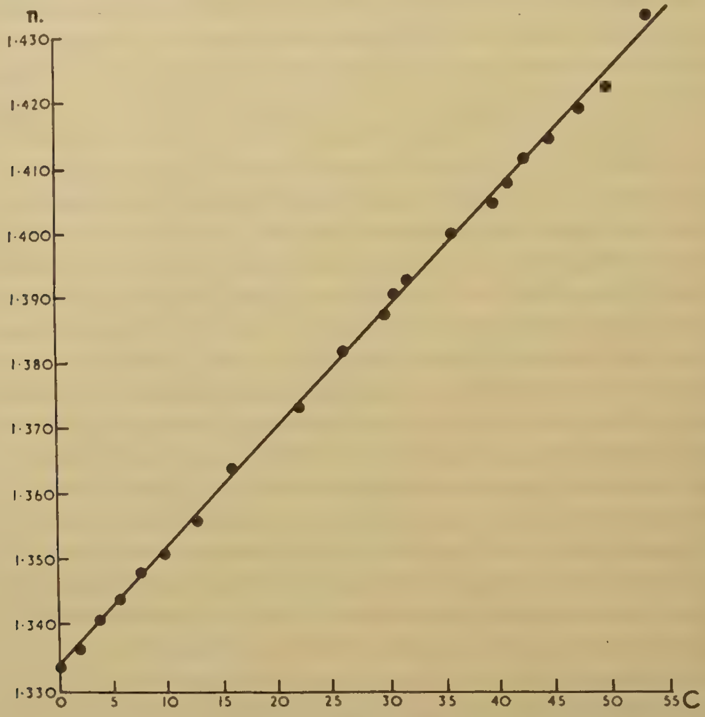

FIG. 2. Variation of refractive index (n) of bovine plasma albumin, fraction V, with co centration (C). From Barer and Tkaczyk (1954).

is negligible at concentrations up to nearly 50 per cent. and is small even 55 per cent., the practical limit of solubility. The value of α, the slope of line, was found to be ·00182. This is in quite good agreement with ot reported values in table 1, but no special accuracy is claimed because of uncertainty in the value of $E_{1\,\text{cm.}}^{1\%}$. The main point of the result is that α is c stant over a very wide range of concentration, so that it is possible to deduce c centrations with considerable accuracy from measurements of refractive ind

(Since this manuscript was prepared a personal communication has b received from Mr. A. W. Kenchington concerning work he has carried out the refraction increment of gelatin. In order to use high concentrations it v necessary to work at a temperature of 34·5° C. It was found that the refract index varied linearly with concentration up to the highest concentration u st over 30 per cent.). The refraction increment was found to be ·0018. This ıbout the same as that found for other proteins, which is of some interest in ·w of the fact that gelatin contains almost no tryptophane or tyrosine and mparatively little phenylalanine. Gelatins from very different collagenous ırces were found to have the same refraction increment, and chemical ·atment such as processing with lime or with acid did not affect the refrac- e index, nor did heating at 100° C. for 1 hour in a sealed tube. The specific raction increment of collagen has been measured recently by M'Ewen and att (1953). They obtained values between ·00189 and ·00192 at 5,780 Å. ıese values are a little higher than those for gelatin and most other un- mented proteins, but collagen is insoluble in water and has to be dissolved dilute acids.)

fect of medium

Putzuys and Brosteaux (1936) and Hand (1935) have pointed out that the lue of α may vary with the nature of the solvent. This is fairly obvious be- use in the limiting case, when the 'solution' consists of pure solute only, the al refractive index attained will be that of the latter and will be independent the solvent. Thus, if we imagine a solute whose refractive index in the solid ıte is 1·6, the refraction increment will be greater in a solvent of refractive dex 1·33 than in one of refractive index 1·50. Differences of this sort are un- ely to be of any importance in the case of constituents of living cells which n be regarded as being dissolved in a very dilute salt solution, but they must taken into account in interpreting values for refraction increment obtained th substances which are only soluble in non-aqueous media or in strong lt solutions.

THE REFRACTION INCREMENT OF CERTAIN CELLULAR CONSTITUENTS

Protein derivatives and constituents

Denatured protein. Barker (1934) measured the refraction increment for tive and heat-denatured egg albumin solutions, using first a dipping refrac- meter accurate to six decimal places, and then an interferometer reading to ght places. The change in α was extremely small. A value of ·001854 was und for the native albumin, and values for the heat-denatured albumin ·re ·001844 and ·001833, measured with the refractometer and interfero- eter respectively. The change was thus approximately 1 per cent. The pH the native solution varied between 4·6 and 5·8, that of the denatured aterial between 6·9 and 7·4, so that it is possible that part of the variation in nay be due to a change in pH. At all events the result is interesting in that it ows how little the refractive index is affected by quite drastic treatment.

Protein digests. As long ago as 1912 Robertson, using a refractometer accu- te to ·000078, found no detectable change in refractive index during the com- ete hydrolysis of a sodium caseinate solution by trypsin. We have ourselves en unable to find any change in the refractive index of a bovine plasma umin solution subjected to digestion by pepsin.

Peptones. In order to obtain some information about the behaviour of mi
tures of protein derivatives, we have measured the refractive indices of pepto
solutions in the concentration range 0–50 per cent. Commercial pepto
preparations differ considerably in their composition and in the amount
insoluble matter which they contain. Evans's bacteriological peptone, whi
is completely soluble in concentrations of 50 per cent. w/v, was used. Th

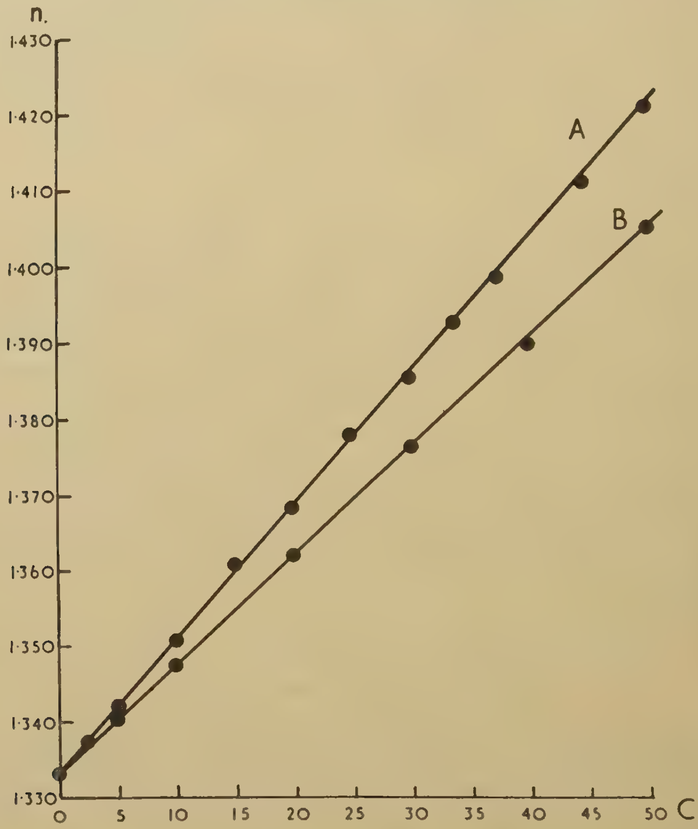

Fig. 3. Variation of refractive index (*n*) of A, Evans's peptone, and B, glucose, with concentration (*C*).

appears to contain mainly relatively small molecules capable of passi
through a collodion membrane. Measurements were carried out at 20° C w
an Abbe refractometer, as already described. The results are summarized
fig. 3. This shows that the refractive index increases linearly with concentr
tion up to 50 per cent. The refraction increment over this range was ·001
This value refers to the powder as supplied by the manufacturers and is n
corrected for water content. The latter is stated to be approximately 5 p
cent., so that the true value of α for the dried material would be ·00183. T
again is within the range of values found for proteins. At all events the val

r peptone can reasonably be taken as applying to a mixture of non-protein trogenous constituents in living cells.

Amino acids. Values of α for four amino acids have been determined experi-entally by Adair and Robinson (1930). Their results were: glycine ·00179, anine ·00171, valine ·00176, and tryptophane ·00252. These values are in cordance with general expectations. Tryptophane contains conjugated ouble bonds and has a high refractivity. The other amino acids contain no onjugated double bonds and their specific refraction increments are slightly wer than those of proteins. Since proteins contain relatively small amounts f tyrosine, tryptophane, and phenylalanine (which contain conjugated double onds) one would expect their specific refraction increment to be only slightly igher than the values for non-aromatic amino acids.

Adair and Robinson showed that there is excellent agreement between the bserved values of $\frac{n-1}{d}$ and the figures calculated theoretically from values or atomic refractivities given in the literature. The only important deviation was in the case of tryptophane. The same authors also calculated the refracti-ity of albumin and found fairly good agreement between the observed and alculated values. The agreement would probably have been even better had t been possible to take into account the effect of other aromatic amino acids part from tryptophane.

Surveying these results it will be seen that there is a remarkable degree of onstancy in the values of α for many types of protein derivatives or mixtures f such derivatives. This is in conformity with the behaviour expected rom the simple theoretical discussion on page 405.

Nucleic acids and nucleoproteins

According to Tennent and Vilbrandt (1943) the refraction increment of odium thymonucleate solutions is ·0016. More recent, unpublished measurements by Cecil (1951) on the possibly purer preparations of Gulland and others (1947) gave higher values approaching ·002. Further work on such compounds is evidently desirable, but it is again clear that these results do not differ very greatly from those for proteins. The only figure available for a nucleoprotein is ·0017, obtained by Oster (1954) for tobacco mosaic virus. This again is close to the values for other proteins.

Lipids and lipoproteins

It is virtually impossible to assign any sensible meaning to the specific refraction increment of most fatty substances because they are of course insoluble in water. The refractive indices of most animal and vegetable fats lie between about 1·45 and 1·48. Thus the values for human fat, horse fat, whale fat, and wool fat are given as approximately 1·46, 1·465, 1·47, and 1·48 respectively. If we calculate the value of α from these figures on the assumption that the fats are perfectly water soluble, we obtain, after making due allowance for the low density (approximately ·93) of fats, a value of about ·0015. This is rath lower than the figures for proteins, but its meaning is obscure. On the who it seems more reasonable to assume that fats will exist in living cells mainly two forms: (*a*) as actual fatty droplets of microscopic size (in which case the presence will scarcely affect the values obtained for refractive index of th cytoplasm, since measurements can be made on regions free from such droplets), and (*b*) combined with protein as lipoproteins. There is an increasin body of evidence in support of this view (see Faraday Society Discussion o Lipoproteins, 1949). According to Oncley and others (1950), 70 per cent. the normal plasma lipids are contained in the β_1 lipoprotein fraction of th plasma proteins. The chemical constitution of this lipoprotein is very remarkable in that it contains approximately 23 per cent. protein, 29 per cen phospho-lipids, 39 per cent. cholesterol esters, and 8 per cent. free cholesterol. In other words, more than three-quarters of the weight of this substanc is accounted for by lipids. Another plasma fraction, α_1 lipoprotein, contain 65 per cent. protein and 35 per cent. lipide. Clearly such complexes coul contain enough lipid to account for the quantities present in living cell Fortunately, figures are available for the specific refraction increment of thes lipoproteins. According to Armstrong and others (1947), the value of α for β lipoprotein is ·00171, for α_1 lipoprotein ·00178. Adair (1952) obtained value of between ·00170 and ·00171 for plasma lipoprotein fractions. It thus appear that the presence of even very considerable amounts of lipids has only small effect on the refractile properties of proteins. Oncley and others remarked that the solubility and amphoteric properties of lipoproteins ar characteristic of the protein fraction rather than of the lipids; the same i apparently true for refractivity. Another piece of evidence which has som bearing on this question is that Armstrong and others found that althoug the figures for α for human plasma from cases with lipaemia were lower tha the normal average of ·001833, even the lowest value did not fall below ·00176. From the point of view of the present work, therefore, it seems improbable that the presence of bound lipids in living cells would be likely t alter the refraction increment by more than a small amount, probably less tha 5 per cent.

Carbohydrates

Refractometry is a standard method of estimating sugar concentration in industry, and the refractive indices of several sugar solutions have been measured in great detail over a very wide range of concentrations. Unfortunately, most of the published tables (see Honig, 1953, and Internationa Critical Tables) give sugar concentrations in terms of grams per 100 grams of solution. With these units the refractive index does not vary linearly with concentration, so that α is not constant. If, however, one recalculates the concentration in terms of grams per 100 ml. of solution, using publishec density tables, it is found that α is constant over a very wide range with very nearly the same value (approximately ·0014) for different sugars. We have ourlves verified this experimentally for glucose, obtaining $\alpha = \cdot 00143$ in the nge 0–50 per cent. concentration (see fig. 3). Such values are distinctly wer than those for proteins and might be a cause of error when attempting to easure solid concentrations of living cells by refractometry. However, as in e case of lipides, it is improbable that all intracellular carbohydrates are resent in simple solution. Some will no doubt occur in the form of glycogen ranules, and some are probably bound to proteins. Unfortunately no figures re available for the refraction increment of glycogen or other polysaccharides r for glycoproteins and mucoproteins, but serum globulins contain about per cent. of carbohydrates. Armstrong and others (1947) obtained values for of $\cdot 00183$ for α_2 globulin and $\cdot 00185$ for β_1 globulin. These results are ot markedly different from those for other proteins. Although there are any gaps in our knowledge of the refractile properties of carbohydrates, it eems on the whole improbable that their presence in moderate amounts ould cause the refraction increment of protoplasm to deviate markedly from hat of pure proteins.

Inorganic constituents

Many investigations have been carried out on the refractive indices of salt olutions, and extensive summaries are given in the International Critical ables. Care has to be taken in using such tables that concentrations must be xpressed in terms of grams per 100 ml. of solution. Baxter and others (1911) ound that the refractive index increased linearly with concentration over a ide range. They found $\alpha = \cdot 0016$ for sodium chloride and $\alpha = \cdot 0012$ for otassium chloride. Other workers have obtained similar results. The figures iven for calcium and magnesium chlorides in the International Critical ables are $\cdot 0011$ and $\cdot 0012$ respectively. Although in most cases the values of for salts are less than those for proteins, the concentration of intracellular alt is usually low, and in any case the immersion medium contains similar alts, so that in general one can probably ignore the contribution to the total efractive index made by intracellular salt.

THE REFRACTION INCREMENT OF PROTOPLASM

At first sight it might appear impossible to suggest a mean value of α for so complex a mixture as living protoplasm. Undoubtedly α must vary to some extent with different types of cells. Fortunately, however, the main solid organic constituent of the cytoplasm of nearly all cells is protein and, as already discussed, at least some of the lipides and carbohydrates are probably present in the form of complexes with protein, and the refraction increments of such complexes do not differ markedly from those of other proteins. Nucleic acids, amino acids, and other non-protein nitrogenous constituents also have very nearly the same refraction increments as proteins. Until more is known about the chemical analysis of living cells and about the forms in which various substances exist in such cells, it seems reasonable to take the mean value of α for protoplasm as ·00180. This is very slightly less tha the average for most proteins and takes into consideration the possib lowering effect of lipides and carbohydrates. For the majority of cells it seem unlikely that this figure would be in error by more than perhaps 5 per cent but of course ignorance of the exact value of α must always remain a fundamental error of the method. It may be that in some cells of very unusu chemical composition the error may be greater, but even if the cell were composed entirely of carbohydrate α would not fall below ·0014. At the othe extreme, so far as we are aware, no values greater than ·0020 have ever bee reported for aqueous solutions of organic substances.

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Fig. 1 is reproduced by kind permission of the Editor of *Nature*.

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ie Changes of Water Distribution in Cytoplasm and uclear Sap during Division as Indicated by Changes in their Refractive Indices

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SUMMARY

The changes in refractive index of the cytoplasm and nuclear sap of the dividing ermatocytes of *Locusta migratoria* were measured by the refractometric technique st employed by Barer and Ross in 1952. It was found that, while certain restricted gions behaved slightly differently, the refractive index of the greater part of the cyto-asm fell during the first part of prophase from 1·354 in the resting cell to about 1·346, d rose again steadily after the beginning of metaphase until it approximated to its iginal value at the end of telophase. These results did not accord very closely ith previous refractometric measurements, but showed a marked agreement with a evious record of changes in viscosity made by Carlson in 1946. It is suggested that is parallel behaviour is best explained by assuming that shifts in water distribution cur in the dividing cell, and that during prophase the cytoplasm and nuclear sap come correspondingly more aqueous as the chromosomes become denser.

INTRODUCTION

THE aim of the investigation was to try to determine the changes in refractive index that occur during cell-division by using the method of live-cell fractometry first employed by Barer and Ross in 1952, which involves the amination by phase-contrast microscopy of cells suspended in varying ncentrations of isotonic protein. Since, however, as was pointed out by arer in 1952, the great majority of the organic substances occurring in the cell ave nearly the same refraction increment (that is to say, their solutions all hibit nearly the same increase in refractive index for each 1 per cent. increase their concentration w/v), such refractometric measurements can be regarded giving a fairly close indication of the water and solid content of the cell and e changes occurring in it.

revious work on the refractometry of dividing cells

Ever since 1882, when Flemming first recorded his impression that cell ivision was accompanied by alterations in the refractive index of the cell, this roblem has, from time to time, aroused interest; but although several subsequent authorities confirmed Flemming's subjective impression, no actual easurements were attempted until 1921, when Vlès made a study of the hanges occurring during the first cleavage division of the fertilized eggs of *chinus.* His method of measuring the refractive index of the cells was to

uarterly Journal of Microscopical Science, Vol. 95, part 4, pp. 425–432, Dec. 1954.]

regard them as spherical lenses and to measure their diameter and focal leng in a parallel beam of light (the focal length depending on the refractive inc of the sphere relative to its surrounding medium). The value he obtained the refractive index of the egg just after fertilization was 1·385, which approximately equal to a 28 per cent. solution of protein and is not an undu high figure in view of the amount of granular yolk that these cells conta During the subsequent division this value did not appreciably change un after metaphase, when, on the appearance of the cleavage furrow, he record a marked increase, to 1·405 or approximately 39 per cent. of protein, follow by a fall to about the previous value on the separation of the blastomer These results have, however, been criticized by Castle, who in 1933 used esse tially the same technique for measuring the refractive index of the mycel stalk of *Phycomyces* in connexion with an investigation of phototropism, a who expressed doubt as to whether the experimental error involved in measu ing the focal distance permitted a refractive index measurement accurate less than 2 units of the second decimal place. But the main disadvantage this technique is that it can only give average values for the whole cell co tents which are in fact rarely wholly homogeneous, least of all during divisio and thus the values obtained may be quite unrelated to the actual refracti index of any one cell-constituent.

Another rather more extensive refractometric study of individual dividi cells was undertaken in 1951 by Pfeiffer, who used an entirely different metho This consisted of inserting into the cell cytoplasm a succession of fine gla micro-needles, each of known refractive index, and judging by the presence absence under the microscope of the Becke line (which characteristical appears at the boundary of two media of different refractive indices), whi kind of glass had a refractive index nearest to that of the cell cytoplasm. H obtained approximately the same values with four widely differing types cell (*Glyceria* pollen mother-cells, *Tradescantia* staminal hair cells, *Ner* eggs, and *Palaemon* glandular tissue), but these did not form any very easi interpretable pattern related to the successive stages of division, and were a very much higher than one might reasonably expect to get with rather wate cytoplasm. Their figures ranged from 1·44 at their lowest, which is equivale to over 60 per cent. of protein, up to over 1·53, which is equal, or nearly equa to the accepted values (quoted by Chamot and Mason, 1938) for the refracti indices of such almost completely dehydrated protein products as leath (1·53), wool, and dried casein (1·54). Consequently, although Pfeiffer stat that in these cases the insertion of the micro-needles did not in any way inte fere with the normal behaviour of the chromosomes, one can hardly avo concluding that the technique must be at fault and probably damages t cells in some respect. The most likely suggestion, made by Dr. R. Barer, that the cell contents in immediate contact with the glass may coagulate a form a thin protein precipitate round the needle, which, while amounting no more than a very local injury, could very well considerably affect t appearance of the Becke line.

e material and method of the present investigation

Spermatocytes from the common locust—*Locusta migratoria migratorioides* proved very suitable for this study because the resting cells were found to splay considerable uniformity of refractive index, the value being equivalent 11 per cent. of protein for both nucleus and cytoplasm in practically every se. The dividing cells, on the other hand, all gave, to a varying degree, lower lues than this. They occurred sufficiently commonly for one to be reasonably rtain of finding several stages in most preparations. In the living material e recognition of each stage with a 4-mm. objective is sometimes difficult—articularly the individual stages of prophase; but while no claim is made to ave established them with complete certainty in every instance, this task as made very much easier through the loan, by Dr. J. R. Baker, of a very fine t of fixed preparations showing all the meiotic stages in the identical material he spermatocytes of *L. migratoria*), which were used as a basis of comparison; nd the existence of these demonstration slides was, of course, a further recommendation for using the material in question.

The technique itself, the theory of which is more fully discussed elsewhere, epends on the fact that when a cell immersed in a protein solution is examined y phase-contrast microscopy (with an ordinary positive, 90° phase-plate), its ontents will give the normal darkish appearance only if the refractive index f the surrounding medium is less than that of the substances within the cell; nd when a more concentrated medium is used with a refractive index greater han that of the cell contents, the latter will appear bright instead. Thus, when one finds a protein medium which approximately *matches* the cell contents in relative brightness, one can assume that the refractive indices of both are practically the same; or, in other words, the concentration of protein outside the cell is then about equal to the concentration of the organic solids comprising the cell protoplasm in question. It was found that by employing this criterion it was possible to detect quite easily refractive index differences of 0·0008, which is approximately equivalent to ½ per cent. of protein.

An essential condition of this technique, of course, is that the protein medium must be isotonic with the cells in question, so that they do not swell or shrink and thus alter in refractive index. This was achieved by dissolving the protein in saline, the correct concentration of which had already been deduced from cell size-measurements on other material. It was found in the present study that a 20 per cent. solution of Armour's bovine plasma-albumin in distilled water caused almost exactly the same amount of swelling of the cells as a 0·2 per cent. solution of sodium chloride. A 20 per cent. solution of the plasma-albumin in 0·7 per cent. sodium chloride solution was therefore used, in order that the total osmotic pressure might be as close as possible to that of the blood of insects (that is to say, approximately of the same osmotic pressure as 0·9 per cent. sodium chloride solution). This albumin-saline solution was diluted with 0·9 per cent. sodium chloride solutions to the various concentrations required, their refractive indices being measured with a Bellingham and

Stanley pocket refractometer. After being dissected out in saline, fragments the testis were teased in a watch-glass containing a relatively large quantity of t medium in question and preparations were made of the resulting cell-suspe sion. (These had the distinct advantage over ordinary wet microscope prepar tions in that they were self-sealing, the protein round the edge of the coversl coagulating almost immediately and preventing further evaporation fro taking place.) A 4-mm. phase-contrast objective was favoured as it facilitat searching and enabled the stages, when found, to be kept continuously und observation; while with a 2-mm. objective they were often moved out of t field through slight surges in the fluid.

Results and conclusions

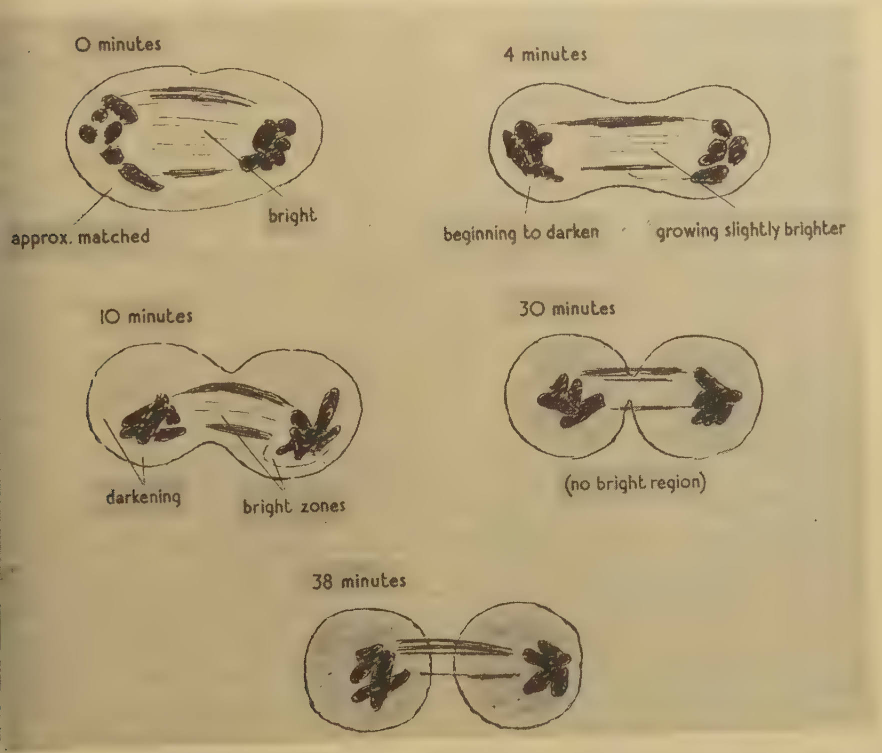
The investigation was very greatly helped by the particularly fortunate fin in one preparation of a cell actively dividing in a 9·5 per cent. protein solutio which was watched continuously over the space of 40 minutes as it progresse from late anaphase through to mid-telophase. This, in addition to providin an important extra piece of evidence in support of the contention that imme sion in isotonic protein cannot greatly upset the cells, enabled all the successiv changes in refractive index at these stages to be recorded, and these are full indicated in the drawing in fig. 1. The remaining measurements, howeve which included the much slower changes that occur in prophase, were derive from single observations and depended on finding and recognizing each stag

The results obtained in the limited time available, which must be regarde as preliminary, are shown in table 1. It will be seen that in spite of a certai number of minor variations (which might be due either to incorrect recog nition of the stage in question or to possible differences between the first an second divisions of meiosis, but which could also be due to individual variatio in different cells), the results shown in the table indicate a reasonably consis tent picture which can be stated as follows:

1. Cell division here is characterized by a marked fall during prophase o the refractive index of the cytoplasm. This reaches a minimum, apparentl at pachytene, when the refractive index can be as much as 0·008 below tha of the resting cell (1·3463). This represents a drop in protein concentratio of about 4·5 per cent. During these stages the nuclear sap frequently appeare appreciably brighter than the surrounding cytoplasm. Ordinarily it is ver difficult to make quantitive deductions about the refractive index of cel inclusions from their appearance under phase-contrast microscopy (see Bare and Tkaczyk, 1954). Here, however, a number of cases were observed i which the nuclear sap still appeared brighter than the background even whe the refractive index of the cytoplasm was closely matched by that of th mounting medium, which suggests that such bright appearances may be trul indicative of a lower refractive index.

2. In the later stages—from metaphase onwards—the refractive index of th cytoplasm appears to rise again fairly steadily, until by late telophase i approximates to its original value, a rise of this kind being certainly observe

the course of the observations covering late anaphase to mid-telophase scribed above. In this case also, a bright region of cytoplasm was found sely associated with the spindle. As this was observed first when the more ripheral cytoplasm approximately matched the background, it again seems obable that the refractive index of this region was lower, which would mean



IG. 1. Successive stages (anaphase to telophase) in a primary spermatocyte of *Locusta migra-ria* actively dividing in 9·5 per cent. Armour bovine plasma-albumin, showing the relative rightness of the cell constituents when viewed with a 4-mm. 90° positive phase-contrast objective. (Semi-diagrammatic; from sketches by S. Tkaczyk.)

hat the cytoplasm within the spindle was relatively more aqueous at this stage. The fact that this region subsequently disappeared while the spindle structure ersisted suggests that it was not just a halo effect due to the latter.)

3. The chromosomes themselves, right from the time of their first appear-ance, always appear as dark bodies in all the concentrations employed and must have a relatively much higher refractive index since they still appear dark even in a 31 per cent. protein solution (refractive index 1·390).

It will be seen that these results do not agree in any way with those of Pfeiffer; nor can they be strictly compared with the rather more credible figures obtained by Vlès, which are average values for the whole cell-contents includ-ing the chromosomes, although they accord with them in certain respects

TABLE I

The appearance, under a phase-contrast microscope, of the cell cytoplasm and nuclear sap of the spermatocytes of Locusta migratoria *at different stages of division when immersed in various concentrations of isotonic bovine plasma-albumin.*

Per cent. Protein	*Resting cell*	*Leptotene/ zygotene*	*Pachytene/diplotene*	*Diakinesis*	*Prometaphase/ metaphase*	*Anaphase*	*Telophase*
11 (R.I. 1·354)	Cytoplasm and nucleus both approximately matched		Cytoplasm and nuclear sap very bright		Cytoplasm very bright		Cytoplasm approximately matched or in some cases still faintly bright
9·5 (R.I. 1·351)	Cytoplasm and nucleus both dark		Cytoplasm and nuclear sap both bright	Cytoplasm faintly dark; nuclear sap bright		General cytoplasm matched or faintly dark — Cytoplasm within spindle bright — Transitory brighter region round chromosomes —	→ became progressively darker → became rather brighter before being occluded → disappeared } continuously observed for 40 minutes
8·5 (R.I. 1·349)	Cytoplasm and nucleus both dark	Cytoplasm dark; nuclear sap faintly bright	Cytoplasm matched; nuclear sap bright	Cytoplasm and nucleus both bright	Cytoplasm faintly dark with a bright region round chromosomes		
6·7 (R.I. 1·346)	Cytoplasm and nucleus both dark	Cytoplasm and nucleus both matched	Cytoplasm and nucleus both approx. matched; in another instance both faintly bright		Cytoplasm dark except round chromosomes where it was approx. matched		Cytoplasm very dark

ich will be discussed. They do, on the other hand, show a noticeable
neral agreement with the changes in cytoplasmic viscosity during mitosis
corded by Carlson in 1946 with the neuroblasts of the grasshopper *Chorto-*
aga viridifasciata. Using the amounts of observable mitochondrial move-
ent as an index of relative viscosity, Carlson made a very careful study of all
gions of the cytoplasm at every stage. His results showed that, in all the
ripheral part of the cell, the viscosity of the cytoplasm falls during the latter
rt of prophase, and, after reaching its minimum at the end of metaphase,
ses during anaphase and regains its original value in the early part of telo-
ase. On the other hand he found that the viscosity of the cytoplasm within
e spindle (which he calls the 'interzonal region') continued to fall through-
t anaphase when the rest was rising. It can of course be argued that a
arallel ought not to be drawn between this work on mitosis and the present
sults obtained from meiotic divisions, but there seems no reason why the
eneral pattern of the *cytoplasmic* changes should be very different in each
se.

One cannot avoid concluding that the most likely explanation for such
arallel changes in both refractive index and viscosity would seem to be in
rms of changes of water distribution within the cell: and indeed these changes
re very much what one might expect to find as the chromosomes begin to
ssume the relatively dense form they have in division: if during prophase
ttle or no water was exchanged between the cell and its surrounding medium,
e condensation of the chromosomes would result in first, the nuclear sap, and
en, as the nuclear membrane became permeable or broke down, the cyto-
lasm, becoming more aqueous; and at a later stage the formation of a rela-
vely solid spindle may account for a related aqueous zone in the ground
ytoplasm. After metaphase, on the other hand, the refractive index of the
ytoplasm increases again before the chromatic material is to any appreciable
egree redispersed, and so it looks as if true synaeresis must be involved at this
tage; and it may be worth noting that it is in fact at about this stage that Vlès
ecorded a significant rise in total refractive index. It is also interesting that
lès specifically mentions a few instances in which he found that his cells
ormed two images at different foci, and this of course is just what one would
xpect to find with his technique when the refractive indices of the cytoplasm
nd nuclear sap were different; and this may sometimes occur, according to
he results of the present investigation.

Finally, it should be emphasized that although these investigations appear
o form a coherent story, the conclusions are in fact based on relatively few
ndings and may quite possibly have to be modified when further work is
ndertaken.

I should like particularly to thank Dr. R. Barer for supervising this work
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The Action of Fixatives on the Ultra-violet-absorbing Components of Chick Fibroblasts

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With three plates (figs. 1, 2, and 15)

SUMMARY

1. This paper describes an investigation by ultra-violet spectrometry of the physical nd chemical changes caused by treating interphase chick heart fibroblasts, grown in issue culture, with a variety of fixatives. The same cells, before and after fixation, were photographed either at 265 and 313 mμ or at a series of wavelengths required for an absorption spectrum.

2. The nucleus of the living interphase cell, apart from nucleoli, is nearly homogeneous, and non-specific light loss measured by the optical extinction at 313 mμ (E_{313}) is not detectable. After fixation the nuclear material is redistributed into filamentous and rounded structures. These nuclear structures are least apparent after osmium tetroxide, methanol freezing-substitution, and freezing-drying; may be more marked after 10 per cent. neutral formalin; and are very marked after Carnoy fixation. E_{313} is measurable in a fixed nucleus and the ratio of optical extinctions E_{313}/E_{265} is always less than about 15 per cent. E_{265} increases as a result of the shrinkage of the nuclear area which ranges from about 8 to 16 per cent. for osmium tetroxide, about 5 to 35 per cent. for neutral formalin, about 19 to 47 per cent. for Carnoy, and about 20 to 40 per cent. for freezing-substitution. Apart from occasional cracks in cells, freezing-substitution and freezing-drying preserved nuclear and cytoplasmic structure as well as osmium tetroxide.

3. (i) Total nuclear extinction is defined as average nuclear extinction times nuclear projected area. Decreases in total nuclear extinction at 265 mμ, after fixation in Carnoy and neutral formalin, range from 10 to 35 per cent. and may be attributed to losses of absorbing material. After formalin fixation there is a decrease of approximately 40 per cent. in the total cytoplasmic extinction at 265 mμ. The method of correcting for non-specific light loss and other possible sources of error are discussed.

(ii) Osmium tetroxide itself absorbs in the ultra-violet and 'stains' the cell, thereby increasing the optical extinction. The staining reaction was studied by fixing films of protein, fat, and nucleic acid.

(iii) After methanol freezing-substitution the total extinction of the nucleus increases slightly.

4. The ratio of optical extinctions E_{265}/E_{280} for the living nucleus is about 2·0 and by applying the methods of curve analysis due to Caspersson it is concluded that the amount of 'standard protein' in the nucleus is small compared with the amount of nucleic acid. After all methods of fixation E_{265}/E_{280} decreases to about 1·3 and similarly it is concluded that the fixed nucleus contains about ten to twenty times as much 'standard protein' as nucleic acid. This curious result is discussed and several possible explanations suggested. It is concluded that the derivation of relative amounts of protein and nucleic acid by analysis of the absorption spectra of either living or fixed cells should be treated with caution.

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CONTENTS

PAG
INTRODUCTION 43
Errors in microspectrometry 43
EXPERIMENTS 43
Material 43
Apparatus 43
Measurements 43
Fixation 43
RESULTS 43
The living interphase cell 43
Formalin fixation 44
Carnoy fixation 44
Osmium tetroxide fixation 44
(i) Fixation of cells 44
(ii) Model experiments 44
Freezing-substitution 44
(i) Fixation of cells 44
(ii) Model experiments 44
Freezing-drying 44
The effect of varying the pH of the culture medium 44
DISCUSSION 45
I. *Total amount of absorbing material in the nucleus* 45
(i) Changes in total amount after fixation 45
(ii) Nature of absorbing substances in the nucleus 45
II. The absorption spectrum of the nucleus 45
(i) The living nucleus 45
(ii) The fixed nucleus 45
III. Comparison of fixatives 45
ACKNOWLEDGMENTS 45
REFERENCES 45

INTRODUCTION

ULTRA-VIOLET microspectrometry pioneered by Caspersson (1936 1950) is used to determine the content of nucleic acids and certain proteins in cells. The aim of such measurements is to relate amounts and concentrations of the absorbing substances in various parts of the *living* cell with the changes occurring during cell metabolism. For many reasons (Caspersson 1950) such measurements have often been made on fixed cells; this introduces the possibility of errors due to physical and chemical changes caused by fixation.

The purely morphological changes due to fixation have been investigated in tissue cultures where the same cell can be observed in the living and undamaged state and again after fixation. From a study of the action of a variety of fixatives on chick fibroblasts observed with dark ground microscopy (Strangeways and Canti, 1927) and salamander macrophages with phase contrast microscopy (Buchsbaum, 1948), it was concluded that osmium tetroxide either alone or combined as in Maximow's fluid, produced the least change in the appearance of the cell. The method of freezing-drying was not investigated.

The chemical changes due to fixation may be studied in several ways. One ıethod is to fix material in bulk and then examine the fixing fluid for new ıemical substances. Sylvén (1951) has analysed the chemical fluids (Carnoy, ›rmalin, &c.), used in fixing various organs of the rabbit and has concluded ıat there is a loss of 10–30 per cent. of the total tissue mass of fresh organs. Iowever, by this method the precise region of the cell from which the losses ccur cannot be determined.

Another method, often used in cytochemistry, is to compare, with the same ɛchnique (staining, for example), cells fixed in a variety of ways. Thus Nurnberger, Engström, and Lindström (1952) have used ultra-violet and ζ-ray microabsorption techniques to compare the ventral horn cells of the pinal cord of the adult cat after fixation in Carnoy, formalin, and by freezing-lrying, and they discuss the difficulties of interpreting their data in terms of he nucleic acid and protein content of the cell. However, in the absence of neasurements on the living cells there is no way of excluding the possibility hat each fixative introduces a common artifact. By using tissue cultures this ›articular difficulty has been avoided and this paper describes an investigaion by ultra-violet microspectrometry of physical and chemical changes ɛaused by treating interphase chick fibroblasts with a variety of commonly ısed fixatives.

rrors in microspectrometry

Errors arise not only in recording the light intensities in the image of the ›bject under the microscope, but also in relating these light intensities to the ımount of absorbing material in the object. The numerous discussions of hese latter errors have been reviewed by Davies and Walker (1953). Some are ɛaused by a heterogeneous distribution of material in the object as is often ›rought about by fixing the relatively homogeneous living cell. It is important herefore to enumerate these errors, since changes in distribution may cause ılterations in the optical extinction of the object, which might be wrongly nterpreted as changes in the amount of absorbing material.

(i) Fixation is often accompanied by shrinkage and hence the concentration ›f material in the cell increases. If Beer's law does not apply, then the change n concentration will lead to an apparent change in the total amount of ıbsorbing material per cell.

(ii) The optical extinction of the cell in the ultra-violet is due partly to real ıbsorption by specific chemical substances and partly to light lost by scatterng, &c., outside the collecting aperture of the objective. This non-specific ight loss is usually assessed by the optical extinction at 313 mμ, at which wavelength the nucleoprotein has no real absorption. Caspersson (1942) sugɡested that the real absorption can be determined by subtracting from the ›bserved optical extinction the optical extinction due to scatter calculated by ɛxtrapolation from the value at 313 mμ by using Rayleigh's law. This law ıolds for scattering particles whose size is about one-tenth of the wavelength ›f light and less, and states that the turbidity or loss of light due to scattering varies inversely as the fourth power of the wavelength. For several reasons this law of scattering cannot be satisfactorily applied to cells. In this investiga tion limits are set to the value of the real absorption at the wavelength 265 m by assuming that the light scatter either varies inversely as the fourth powe of the wavelength (λ^{-4}) or is independent of the wavelength (λ^{0}). It is likel that the true value lies somewhere in between.

(iii) The optical extinction of a volume in which the material is heterc geneously distributed may be less than if the same quantity of material we distributed with no variation in concentration throughout the same volum This is commonly called the 'distributional error' and it can be shown (e. Glick, Engström, and Malmström, 1951) that the magnitude of the errc increases with the optical extinction of the absorbing structures in the cel An experimental approach to this problem has been adopted in this investiga tion.

(iv) As a result of glare, that is, light scattering by surfaces in the objectiv &c., a fraction of the light energy from each point in the object plane is sprea all over the image plane. This causes the measured optical extinction of th object to be lower than it really is and the magnitude of the error increase with the optical extinction. Such an increase in optical extinction usuall occurs as a result of shrinkage due to fixation. Hence glare might cause a apparent decrease in the total amount of absorbing material per cell afte fixation.

EXPERIMENTS

Material

Fragments of heart from 11 to 12-day old chick embryos were grown in fluid medium by a modification of the hanging drop method devised by H. I Fell for cytological studies. After 24–48 hours' growth, when the explant wa surrounded by a single layer of cells, the 0·1 mm. quartz coverslip wa mounted on the culture chamber, a stainless steel ring (1 mm. thick and 20 mn diameter) sealed with a 0·5 mm. quartz slide. (For details see Davies, 1952

Apparatus

The ultra-violet microspectrometer was especially designed for work o living cells and full details of its construction and mode of operation hav been given elsewhere (Walker and Davies, 1950). Briefly, it had the followin features. The cells could be observed continuously with visible phas contrast illumination, provided by an auxiliary optical train, while growin and dividing on the warm stage of the microscope. Without changing th objective an ultra-violet photomicrograph at one monochromatic wavelengt between 248 and 313 mμ could be taken rapidly in about 1 second aft accurate focusing in visible phase-contrast. A series of photomicrographs ten selected ultra-violet wavelengths could be taken in about 90 seconds. Tl objective was a lithium fluoride, fused-quartz achromat of numerical apertu N.A. 1·24, focal length 2·2 mm., designed by R. J. Bracey of the Britis Scientific Instruments Research Association; the condenser was a reflectir stem with N.A. 0·6 and 40 per cent. central obstruction (Seeds and Wilkins, 949). The shadow of a calibrated rhodium step-wedge adjacent to each otomicrograph was used to calibrate the film at each wavelength. At a agnification of ×114 on Kodak Microfile Pan 35 mm. film exposure times ere about 1 second at 265 mμ; film was developed in Kodak D.19.b for 4 inutes at 20° C.

easurements

Two or three cells, with a clear area around them to facilitate accurate ensitometry were photographed in each culture at wavelengths 313 and 55 mμ. The culture was then rapidly removed from the microscope, fixed one of the ways described below, and two further photomicrographs taken f each cell. Measurements were mainly confined to the area occupied by the terphase nucleus (see *Discussion*, I (i) (*a*)). The average optical extinctions 313 and 265 mμ were computed from six to ten traces taken across each ucleus with a recording microdensitometer constructed by P. M. B. Walker 1953). The aperture in the densitometer corresponded to a circle 0·2–0·4μ iameter in the cell. The error in recording optical extinction in a single trace as ±0·005, owing to uncertainty in the background density of the cell, and e error in the average extinction was correspondingly less. The *total xtinctions* of the nuclei (excluding the nucleoli) were determined from the roducts of average optical extinction and projected nuclear area.

The total amount of material absorbing at 265 mμ in the living nucleus was etermined from the expression $E_{265}.A/k$ mg., where E_{265} is the average optical xtinction, A the area in cm.^{2}, and k an extinction coefficient equal to the ptical extinction of the nuclear material at a concentration of 1 mg./c.c. in path length of 1 cm. k was chosen as 20, which is the value for a dilute olution of desoxyribose nucleic acid (DNA) and amounts of nuclear material ere expressed as 10^{-12} gm. DNA. This procedure has been arbitrarily dopted for convenient comparison with the results of other workers for the NA content of cell nuclei. However, in these living nuclei absorbing substances other than DNA, and with different extinction coefficients, may be resent.

Measurements of total amounts of cytoplasmic material tend to be inccurate and laborious owing to the presence of refractile structures and the neven outline. Such measurements were only attempted before and after ormalin fixation.

The complete series of photomicrographs required for an absorption pectrum were taken of only one cell per culture before and after fixation. bsorption spectra were constructed from the average optical extinctions long the same line in the images of the cell at the different wavelengths. To ncrease the accuracy, the average extinction of three traces each separated by pproximately 1μ, was used.

Living cells were also observed and photographed in media of different ydrogen ion concentrations. The coverslip bearing the cells was separated from a quartz slide by two narrow glass strips 0·5 mm. thick. The ends we left open for irrigation with either Tyrode solution or a 1 per cent. soluti of sodium chloride to which hydrochloric acid had been added to reduce pH to 4·6; the solutions were drawn through by filter paper.

In model experiments films of protein, nucleic acid, and fat, were spre evenly on quartz slides cut to fit the Hilger photoelectric spectrophotomete These films were allowed to dry in air and absorption spectra were record before and after fixing them in certain ways.

Fixation

The coverslip bearing the cells was first washed in Tyrode solution 37° C. for about 15 seconds to remove the liquid medium in which the ce were grown. This medium contains proteins which would be precipitated certain fixatives, thereby obscuring cell detail and making quantitati measurements difficult. Cells observed in Tyrode for periods of up to half hour remained perfectly normal in appearance so that the preliminary was ing does not upset the cells. The cells were then fixed in one of the followin ways:

(i) With 10 per cent. neutral formalin-Ringer for 10 minutes (solution pr pared by diluting commercial formalin 1:9 with Ringer; the commerci material was neutralized by shaking with powdered calcium carbonate washed ½ to 1 hour; immersed in water or glycerol.

(ii) With Carnoy's original solution, freshly prepared 1 part of glacial acet acid and 3 parts of absolute alcohol, for 10 minutes; washed twice with per cent. alcohol and either taken down through the alcohols to water ar measured in water or glycerol or immersed in glycerol from absolute alcoho

(iii) By exposure for 2 minutes to the vapour of a freshly prepared 2 per cen solution of osmium tetroxide; washed ½ to 1 hour, immersed in water glycerol; some cells were bleached by Mayer's chlorine method (in Boll Lee, 11th ed., p. 242) for periods of up to 2 hours.

(iv) By freezing-substitution (Simpson, 1941). After the cells had bee washed in warm Tyrode, the surplus solution was drawn off with filter pape The coverslip was then mounted on a brass U-shaped holder either with without a copper plate, about ¼ mm. thick, backing it. The holder was the rapidly immersed and stirred in a bath of propane cooled with liquid nitrogen

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FIG. 1 (plate). Photomicrographs of interphase chick heart fibroblasts in tissue cultur taken with an objective of N.A. 1·24, condenser N.A. 0·6, λ 265 mμ.

A, living cell.

B, after fixation in 10 per cent. neutral formalin; mounted in water.

C, living cell.

D, after fixation in the vapour of osmium tetroxide; mounted in water. Shows a gener increase in light-absorption, especially by the nucleus and centrosphere.

E, cell fixed in 10 per cent. neutral formalin, showing a bleb (arrow).

F, cell fixed directly in methanol; mounted in glycerol. Shows marked precipitation through out the cell.

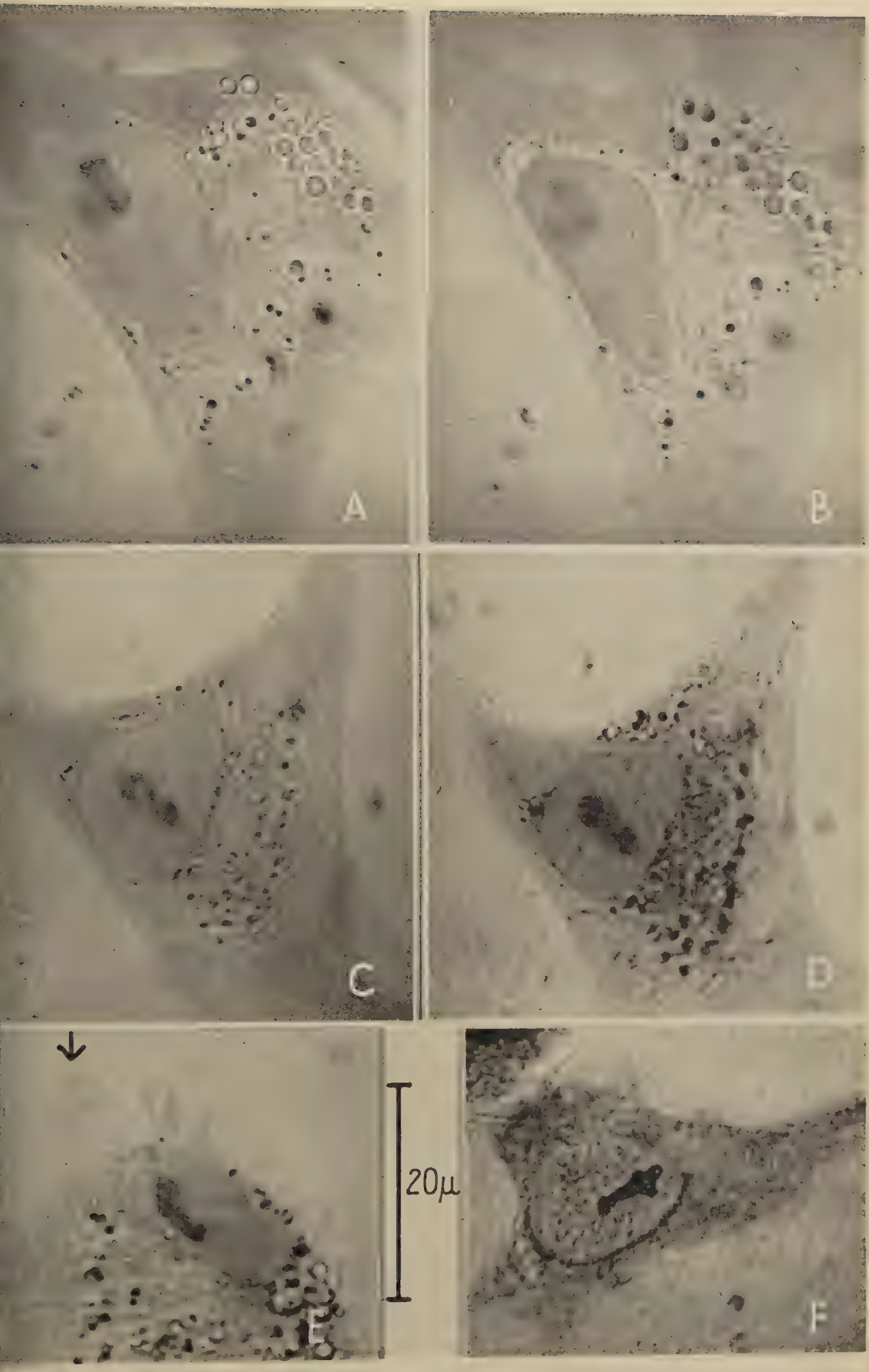


FIG. 1

H. G. DAVIES

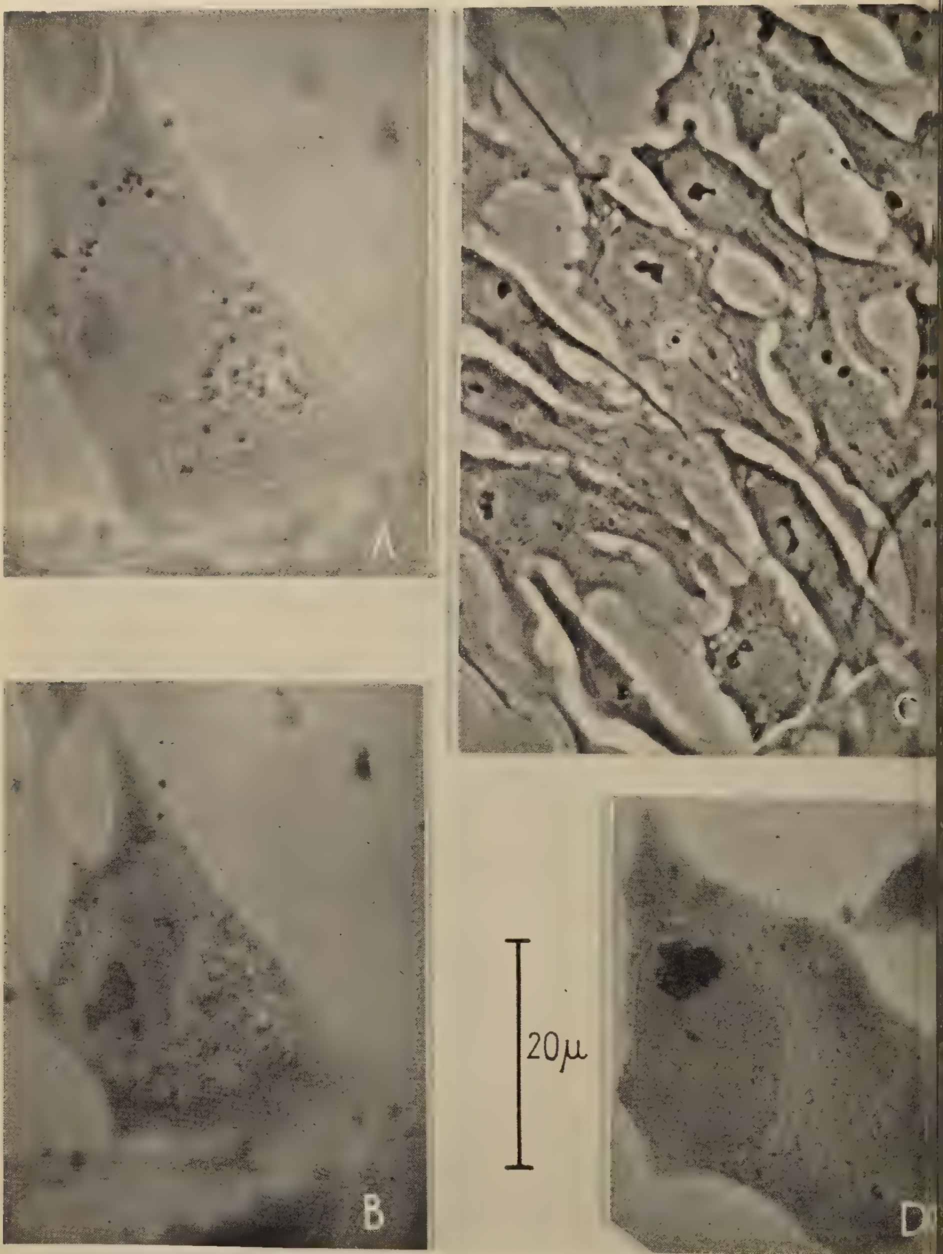


Fig. 2

H. G. DAVIES

ropane, which remains liquid at the temperature of liquid nitrogen, was ggested by Bell (1952). The mounted coverslip was rapidly transferred to a rge glass tube (about 200 c.c.) of methyl alcohol (BDH solvent methanol) reviously immersed in a cold bath of solid carbon dioxide and industrial irits. The tube was removed to a lagged container and allowed to warm up room temperature, which took ½ to 1 hour; loss of water occurred somewhere etween −78° C. and room temperature. The cells were immersed in doubly istilled glycerol.

(v) Some cells were examined after freezing-drying in an Edward's comercial freezer-drier modified to take the 1-inch square coverslip. They were ooled as in freezing-substitution and dried in a vacuum of approximately ·005 mm. for 2 days at about −40° C. Temperatures were measured by a opper-constantan thermocouple in contact with the copper plate backing the overslip. The temperature of the coverslip was raised to about 20° C. before reaking the vacuum so as to prevent condensation of water on to the cells, hich were then immersed in doubly distilled glycerol.

RESULTS

he living interphase cell

The morphological features demonstrable in photomicrographs at 265 mμ f the living interphase cell (figs. 1, A and C, 2, A, also 15, A (see p. 450)) will be numerated briefly. The nucleus, apart from one or two nucleoli, and an ccasional chromocentre, is a nearly homogeneous structure; sometimes ilamentous bodies which lie near the limit of resolution may be seen. In the ytoplasm, which is homogeneously absorbing, the centrosphere can often e distinguished as a less absorbing region adjacent to the nucleus. Usually his area is the site of an aggregation of thin filamentous mitochondria, which, ogether with the fat-droplets, are the most conspicuous structures in the ytoplasm.

Ultra-violet absorption spectra of the living nucleus (figs. 3, A, 5, A, 7, A, 0, A and B) have an absorption maximum in the region 265 mμ. A negligible ptical extinction can be detected at 313 mμ; a slight non-specific light loss ccurs at the boundaries of nucleolus and nucleus and these regions are xcluded from the measurements. The shape of the absorption spectrum etween 270 and 280 mμ varies from cell to cell. Some (fig. 3, A) show a lateau in the region 270–275 mμ, whilst others (figs. 5, A, 7, A) show a plateau

FIG. 2 (plate). Photomicrographs of interphase chick heart fibroblasts in tissue culture. The optical arrangement was the same as in fig. 1, except that fig. 2, C was taken with a Beck phase-contrast objective, N.A. 0·85, focal length 4 mm., λ 546 mμ.

A, living cell.

B, after fixation by methanol freezing-substitution. Shows some cracks.

C, phase-contrast photomicrograph of cells fixed by methanol freezing-substitution. Shows some cracks.

D, cell fixed by freezing-drying; mounted in doubly distilled glycerol.

The scale refers to A, B, and C; D is at one-half of this magnification.

in the region 275–280 mμ. It would be interesting if the stage in interpha were partly characterized by the shape of absorption spectra. However, it not possible to attach much significance to small variations in these spect owing to the inaccuracies of the photographic method of recording and to t limited number of wavelengths employed.

When two substances with known absorption spectra are present in mixture then the relative amounts of the substances may be determined

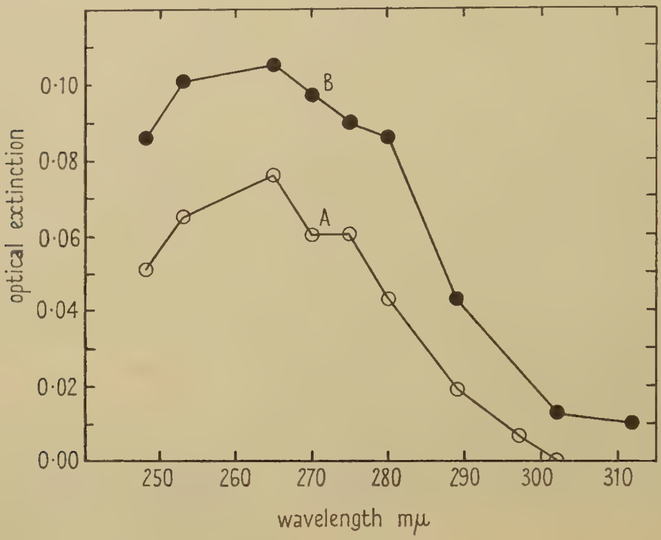
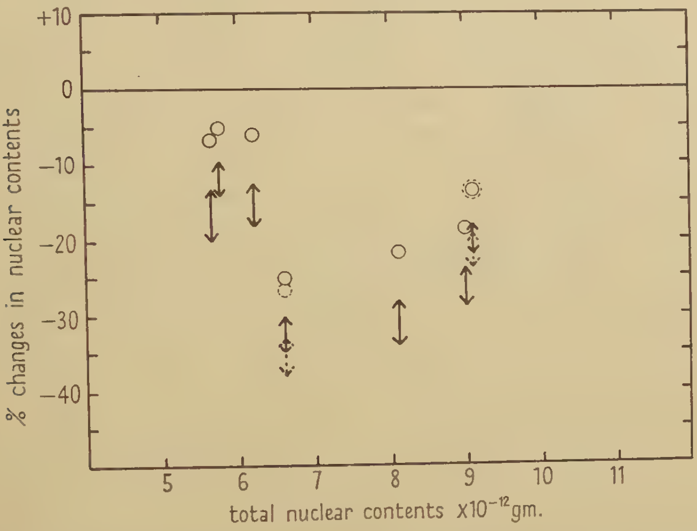


FIG. 3. Absorption spectra of the nucleus of the living cell (A), and after fixation in per cent. neutral formalin (B). Cell mounted in water.

analysis of the absorption spectrum of the mixture. This well-known metho has been used by Caspersson (Caspersson and Santesson, 1942) to determin the relative amounts of nucleic acid and 'standard protein' in the cell fro the ratio E_{260}/E_{280}, where E_{260}, E_{280} are the optical extinctions at 260 an 280 mμ. The 'standard protein' has a certain assumed content of the amin acids tyrosine and tryptophane. For nucleic acid alone, E_{260}/E_{280} is 1·83 an when protein is present the ratio decreases. For technical reasons the rati E_{265}/E_{280} has been recorded in these investigations but the values will n differ so significantly from those at the first-mentioned wavelengths as affect the limited conclusions drawn. The ratios E_{265}/E_{280} for the nuclei several living cells (table 1, col 3: see page 456) give a range of values wit an average approximately 2·0. If the equations of Caspersson and othe (1942) were applied to interpret this value, then it would be concluded th the amount of protein in the nuclei of these living cells is small compare with the amount of nucleic acid. This result is discussed later especially relation to the marked change in the ratio E_{265}/E_{280} which occurs as a resu of fixation.

rmalin fixation

After fixation with 10 per cent. neutral formalin a finely filamentous and anular structure can usually be distinguished in the nucleus (fig. 1, B). The ojected area of the nucleolus sometimes increases but that of the nucleus variably decreases; in the nuclei measured the decrease ranges from 5 to per cent. Changes in the thickness of the nuclei were not measured. The toplasm usually remains homogeneous but occasionally a fine precipitate formed in some regions; mitochondria can still be seen.



IG. 4. Per cent. changes in the total extinction at 265 mμ of the nuclei after fixation in o per cent. neutral formalin. Abscissae: the total amount of absorbing material in the living uclei expressed as DNA. Ordinates: per cent. changes relative to the living cell (zero rdinate). For cells immersed in water the changes are shown by empty circles, non-specific ight loss being neglected; by the upper arrows light loss being corrected according to λ^0, and y the lower arrows when corrected according to λ^{-4}. The dotted circles and arrows show corresponding changes for some cells immersed in glycerol from water.

A characteristic feature is the formation of 'blebs', balloon-like structures containing material faintly absorbing at 265 mμ; they sometimes remain attached to the cell (fig. 1, E) but are usually lost when the culture is washed. Such blebs, formed during formalin fixation, have been noted by others (Buchsbaum, 1948; Crawford and Barer, 1951). After fixation a reduction in the optical extinction at 265 mμ of the cytoplasm is apparent even in photomicrographs, and under positive phase contrast the cytoplasm appears less dark.

The total amount of cytoplasmic material absorbing at 265 mμ was found to have decreased on an average by approximately 40 per cent. after fixation; six cells were measured. Clearly some if not all of the loss in absorption occurs by bleb-formation.

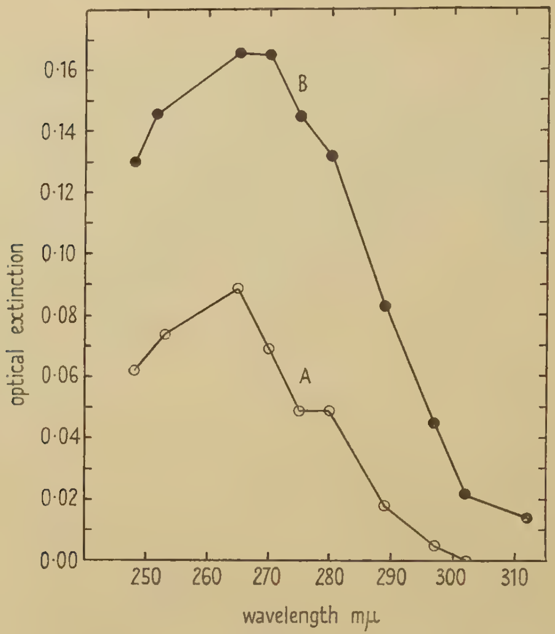


Fig. 5. Absorption spectra of the nucleus of the living cell (A), and after fixing in Carnoy (
Cell mounted in glycerol.

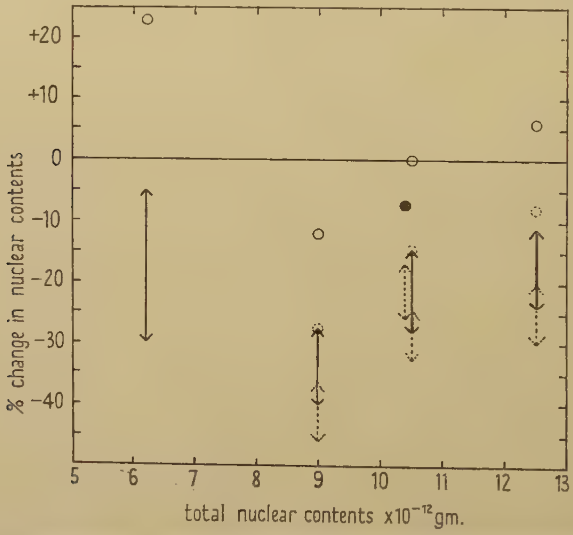


Fig. 6. Per cent. changes in the total extinction at 265 mμ of nuclei after fixation in Carno
Abscissae: the total amount of absorbing material in the living nuclei expressed as DN
Ordinates: per cent. changes relative to the living cell (zero ordinate). For cells immersed i
water the changes are shown by empty circles, non-specific light loss being neglected; by th
upper arrows when light loss is corrected according to λ^0, and by the lower arrows when co
rected according to λ^{-4}. The dotted circles and arrows show corresponding changes for som
cells immersed in glycerol from water and the full circle and dotted arrow for a cell immerse
in glycerol after absolute alcohol.

fter fixation the optical extinction of the nucleus at 313 mμ becomes asurable and is attributed to a non-specific loss of light. The ratio of the ical extinctions at 313 mμ and 265 mμ, E_{313}/E_{265}, in the fixed cell can be veniently used as a measure of this non-specific loss; it is about 7 per cent. a result of shrinkage the optical extinction of the nucleus at 265 mμ reases markedly as shown in the comparative absorption spectra (fig. 3, A l B). After fixation there is a significant drop in the ratio E_{265}/E_{280} to a ue which is nearly independent of the way in which the optical extinctions corrected for non-specific light loss (table 1, cols. 4, 5, 6: see page 456). In fig. 4 the difference between the total nuclear extinctions of the living l fixed cells are expressed as percentages of the total extinctions of the ing nuclei. The circles denote the changes when the non-specific loss is not en into account and the upper and lower arrows the change when the non-ecific loss is assumed to vary as λ^0 or λ^{-4} respectively. The ratios E_{313}/E_{265} the fixed cells are given approximately by subtracting the values for the per arrows and the corresponding circles; the exact values could be calcu-ed by simple algebra. There is very little further change when cells are mersed in glycerol (dotted data). It is concluded that as a result of fixation ere is a decrease in the total nuclear extinction ranging from 10 to 35 r cent.

rnoy fixation

After fixation in Carnoy the material in the nucleus is redistributed into anules (fig. 15, B, p. 450); shrinkage of the nuclear area ranges from 20 to per cent. The nucleolus shrinks, the mitochondria are destroyed and the toplasm becomes granular. The non-specific light loss is now greater than er formalin fixation; E_{313}/E_{265} ranges from 10 to 14 per cent. The ratios $_{65}/E_{280}$ (table 1) deduced from the comparative absorption spectra (fig. A and B) show a similar change to that found after formalin fixation.

Fig. 6 shows that there is a measurable decrease in the nuclear non-specific ht loss when cells are transferred from water to glycerol, but little further ange in the total extinction. It is concluded that the decrease in total clear extinction is similar in magnitude to that after formalin fixation.

smium tetroxide fixation

(i) *Fixation of cells.* When fibroblasts are fixed in the vapour of osmium roxide there is little morphological change. Any nuclear detail is somewhat hanced and the area of the nucleus shrinks by 8–16 per cent., the cyto-asm remains homogeneous and the mitochondria are well preserved. How-er, a general increase in the optical extinction at 265 mμ is apparent even the photomicrographs (fig. 1, D). Fat-droplets become highly absorbing d the controsphere stands out as a more absorbing region; mitochondria so may be more easily distinguished. After fixation the absorption spectrum the nucleus (fig. 7, B) shows a relatively large increase in optical extinction 313 mμ, and a continual increase towards shorter wavelengths.

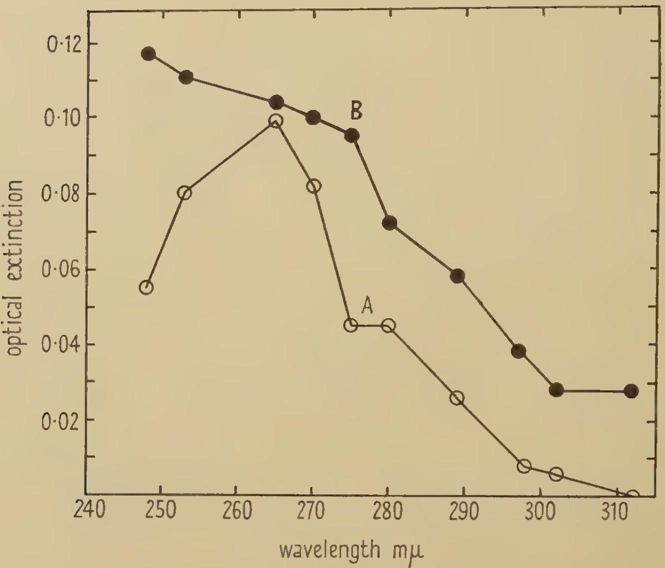


FIG. 7. Absorption spectra of the nucleus of the living cell (A), and after fixation in the vapo of osmium tetroxide (B). Cell mounted in water.

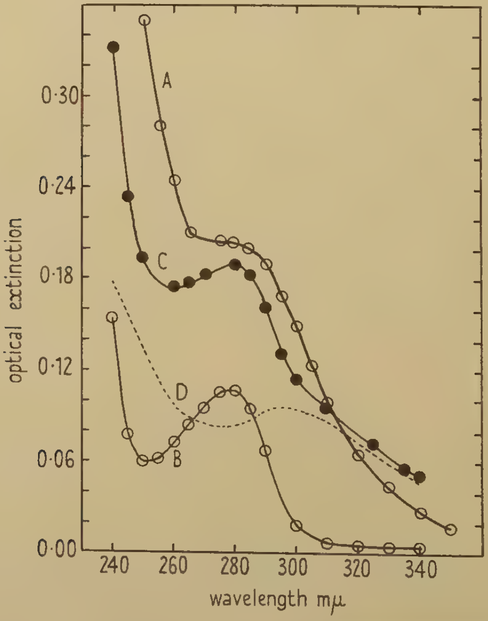


FIG. 8. A, absorption spectrum of a 0·005 per cent. solution of osmium tetroxide (1 cm. pa length). B, absorption spectrum of an air-dried film of egg-white. C, the same film af exposure to the vapour of a 2 per cent. solution of osmium tetroxide for 2 minutes. D, absor tion spectrum of reduced osmium tetroxide obtained by subtracting curve B from curve

ii) *Model experiments.* The possibility that the fixative itself stains the cell, t is, combines with the cell substances to produce additional absorption, was ted by model experiments. Osmium tetroxide in dilute solution absorbs preciably (fig. 8, A) and a film of egg-white exposed to its vapour showed a rked increase in ultra-violet absorption (fig. 8, B and C). The spectrum g. 8, D) obtained by subtracting curve B from curve C is different from that osmium tetroxide and probably represents a reduced form. Films of latin and bacon fat showed similar increases in absorption after exposure the vapour of osmium tetroxide and by subtraction similar spectra were tained for the reduced form of osmium tetroxide. However, a film of ymus nucleic acid similarly exposed showed no change in absorption. Apart om explaining the general increase in ultra-violet absorption of the fixed ll, these observations may also be helpful in the interpretation of electron icrographs where the contrast may be partly due to differential staining ter fixation with osmium tetroxide.

The ratio E_{265}/E_{313} for the reduced form of osmium tetroxide was found to approximately 1·1. This value was used to correct the optical extinction the cell at 265 mμ from the optical extinction at 313 mμ. The results tained for the changes in total nuclear extinction are described in detail the text to fig. 9. The optical extinction of the cell increases after washing ith water for half an hour; this may be due to a slow reaction between the xative and the cell. The nuclear extinction at 313 mμ was unchanged as a sult of immersion in glycerol and could not be completely removed by eaching. There was fair agreement between the corrected decreases in total uclear extinction for cells after washing and after bleaching. These results dicate in a general way how the absorption of the nucleus varies after xation with osmium tetroxide vapour, but the method used in correcting or the additional absorption is probably not very reliable.

reezing-substitution

(i) *Fixation of cells.* After fixing by freezing-substitution with methyl cohol the nucleus either appears homogeneous or contains fine structure ear the limits of resolution of the ultra-violet microscope. The ground sub- ance of the cytoplasm also is well preserved and often appears homogeneous though a fine reticular structure may sometimes be seen; the mitochondria nd fine cytoplasmic processes appear almost unaltered (fig. 2, B). There is n overall shrinkage of the cell, that of the nuclear area ranging from 20 to 0 per cent. The tissue culture explant also contracts and this results in a gap etween it and the sheet of cells. Cracks also appear running through the cells. owever, only a small fraction of the cells are cracked and the appearance of e fixed cells shown in the phase-contrast photomicrograph (fig. 2, C), is very felike.

Cells fixed by freezing-substitution contrast sharply in appearance with ells fixed directly in methyl alcohol (fig. 1, F) where an intense precipitate is

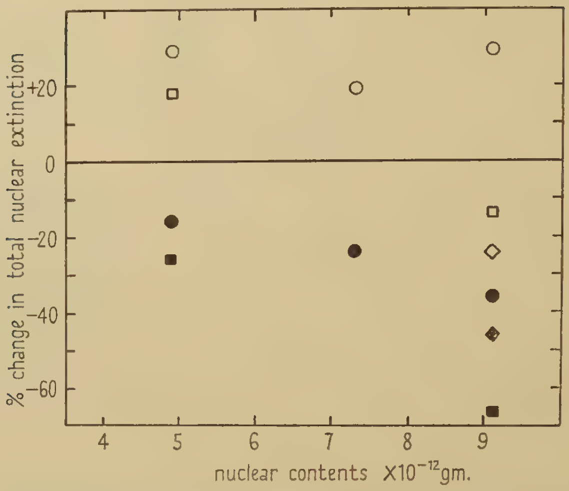


FIG. 9. Per cent. changes in total extinction at 265 mμ of the nuclei after fixation in the vapo of osmium tetroxide. Abscissae: the total amount of absorbing material in the living nucl expressed as DNA. Ordinates: per cent. changes relative to the living cell (zero ordinat The optical extinction at 265 mμ is corrected from the extinction at 313 mμ by usin E_{265}/E_{313} = 1·1, obtained from fig. 9, D. The empty and full squares indicate the uncorrect and corrected values respectively before washing. The empty and full circles indicate th uncorrected and corrected values respectively after washing. The empty and full diamon indicate the uncorrected and corrected values respectively after attempts to bleach the ce

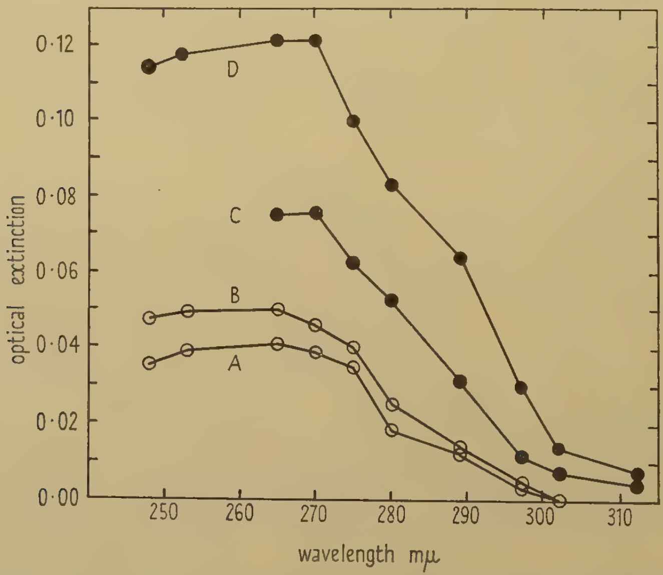
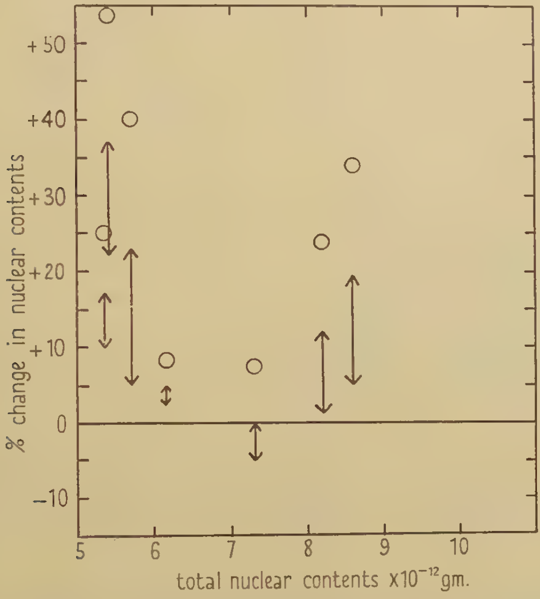


FIG. 10. Absorption spectra of the nucleus of living cells (A, B) and the corresponding spect (C, D) after methanol freezing-substitution; cells mounted in doubly distilled glycerol. A an C correspond.

n throughout the cell and the mitochondria are mostly obliterated. Pre- nably this is the result of diffusion currents, absent during freeze-sub- ution, which sweep the material in the cell to new positions (Bell, 1952). The first step in freezing-substitution, the attempt to vitrify the cell, is the ne as that in the better-known freezing-drying method of Altmann and Gersh, iewed by Bell (1952). One important difficulty sometimes encountered in ezing-drying has been the formation of ice crystals within the cell, giving



G. 11. Per cent. changes in the total extinctions at 265 mμ of nuclei fixed by methanol ezing-substitution; cells in doubly distilled glycerol. Abscissae: the total amount of absorb- material in the living nuclei expressed as DNA. Ordinates: per cent. changes relative to living cell (zero ordinate), are shown by empty circles, non-specific light loss being glected, and by the upper and lower arrows when light loss is corrected according to λ^0 and λ^{-4} respectively.

e to redistribution of material around the boundaries of the crystals. Such ystals can arise either through imperfect vitrification during cooling or rough devitrification before or during dehydration. The ice crystal artifact ls noted during early attempts at freezing-substitution in the present vestigation and was traced to devitrification of the cells during transfer om the propane to the cooled methyl alcohol. The coverslip bearing the lls has only a small thermal capacity and when in air quickly warms up yond the devitrification temperature of the cell. A thin copper plate at the ck of the coverslip, although it hindered cooling, usually prevented notice- le ice crystals from forming, but despite this precaution a small proportion the cells (probably about 10 per cent.) localized in zones of the culture often owed a marked reticular structure.

The non-specific light became measurable after fixation, the ratio E_{313}/E ranging from 3 to 12 per cent. Comparative absorption spectra of the nucl showed a decrease in the ratio E_{265}/E_{280} (table 1). The total nuclear exti tions at 265 mμ (fig. 11) increased after fixation and the possibility of fixative staining the cell was tested.

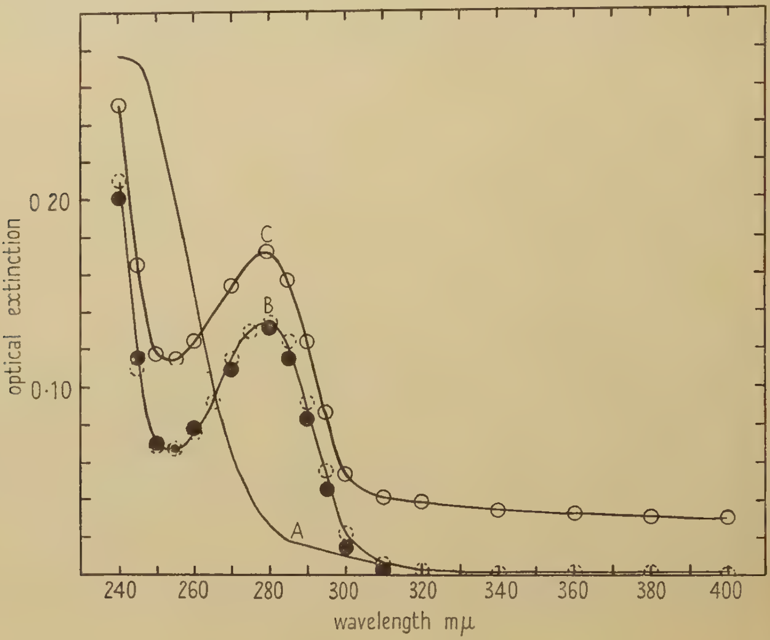


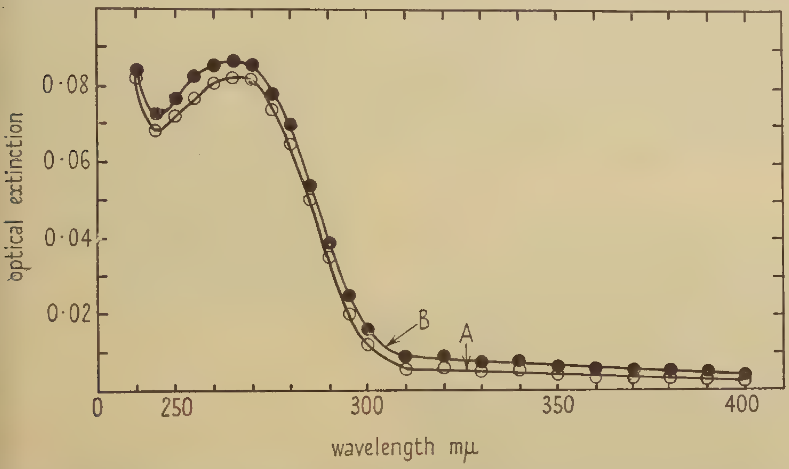
FIG. 12. A, absorption spectrum of 0·25 per cent. solvent methanol (1 cm. path leng B, absorption spectrum of air-dried film of egg-white, broken circles. C, absorption spectr of the film after methanol freezing-substitution. The full circles on spectrum B are obtai by correcting C for non-specific light loss.

(ii) *Model experiments.* The solvent methanol had a high ultra-vio absorption (fig. 12) and the optical extinction of a film of egg-white increas after freezing-substitution (figs. 12, B and C). The fixed and dried film cracked so that it no longer looked transparent in ordinary light and increase in extinction was attributed to light scatter. The extinction at wa lengths greater than 313 mμ was found to vary inversely as the waveleng and the remainder of the spectrum was corrected by extrapolation. The c rected extinctions (full circles) lie, within the limits of experimental error, the original spectrum for egg-white.

In a similar experiment a film of ribonucleic acid mixed with egg-white fixed by methanol freezing-substitution (fig. 13, A and B). This film was mu thinner, exhibiting interference colours, and there were no cracks in the fix and dried film. The increased extinction was small, approximately the sa at all wavelengths, and must be attributed to non-specific light loss in natured egg-white. These experiments make it less likely that the increase total nuclear extinction is due to the fixative staining the cell.

eezing-drying

After freezing-drying, the tissue culture mounted in glycerol is similar in pearance to a culture after alcohol freezing-substitution. The nucleus of the ell-fixed cell either appears homogeneous or contains a very fine reticular ructure. Well-preserved mitochondria lie in a cytoplasm in which structure often difficult to detect (fig. 2, D). Cells sometimes show cracks and occa- nally a marked reticular structure due to the formation of ice crystals.



G. 13. A, absorption spectrum of a film obtained from a mixture of egg-white and ribonucleic d. B, the same film after methanol freezing-substitution, showing a slight increase in non-specific light loss.

Frozen-dried cells appear to be less firmly attached to the coverslip than emically fixed cells. Thus the edges of cells mounted in glycerol were some- nes seen to have curled away from the coverslip and the explant also became tached from the glass, usually when the glycerol was dropped on to the ozen-dried culture. The loss of the explant, a convenient landmark, made difficult to find the same cells after fixation. Principally for this reason no sults are presented here on the change in total nuclear absorption after eezing-drying.

A typical absorption spectrum (fig. 14) of the nucleus of a frozen-dried cell owed a significantly lower ratio E_{265}/E_{280} (table I) than is characteristic of e living cell.

he effect of varying the pH of the culture medium

Lewis (1923) has described how homogeneous nuclei of cells in tissue ulture become filled with refractile structures when the pH of the medium surrounding the cells is reduced to about 4·6. When the pH is returned normal the material in the nucleus, under certain circumstances, reverses the homogeneous state. Measurements of the total nuclear extinction at ea of these three stages would seem to be a good way of checking the magnitu of any error due to distribution (see Introduction (iii)). This experiment w suggested to the writer by A. E. Mirsky, who has observed similar reversib changes in the distribution of material in isolated nuclei (Ris and Mirsk 1949).

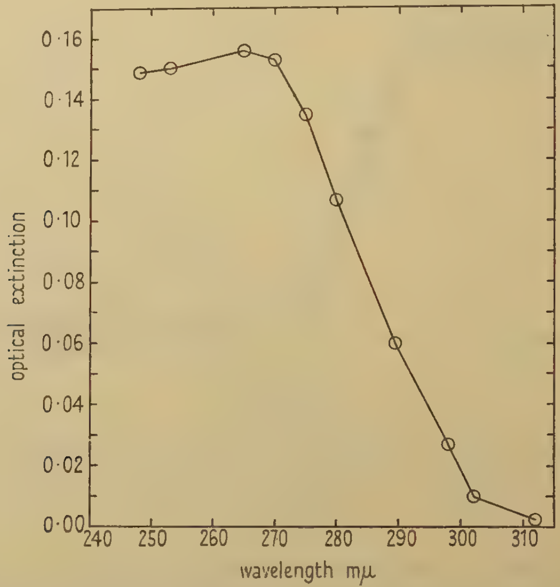


FIG. 14. Absorption spectrum of the nucleus of a cell frozen-dried and mounted in doub distilled glycerol.

Cells were photographed at 265 mμ (fig. 15, C) and 313 mμ immediate after the protein medium had been washed away with Tyrode. The Tyro was then replaced by the saline at pH 4·6; 5–10 minutes later the moveme of the mitochondria had ceased. In about ½ to 1 hour, during which time t culture was occasionally re-flooded with the acid medium, the material in t nucleus aggregated into refractile threads and granules. The cells we immediately photographed at 313 and 265 mμ (fig. 15, D) and then flood

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FIG. 15 (plate). Photomicrographs of interphase chick heart fibroblasts in tissue cultu Optical arrangements as in fig. 1.

A, living cell.

B, after fixation in Carnoy; cell mounted in glycerol. There is marked precipitation throug out the cell.

C, living fibroblast immersed in Tyrode.

D, same cell as C at pH 4·6; the nucleus is full of threads and granules.

E, same cell after subsequent immersion in Tyrode, when the nucleus reverts to its previo homogeneous state.

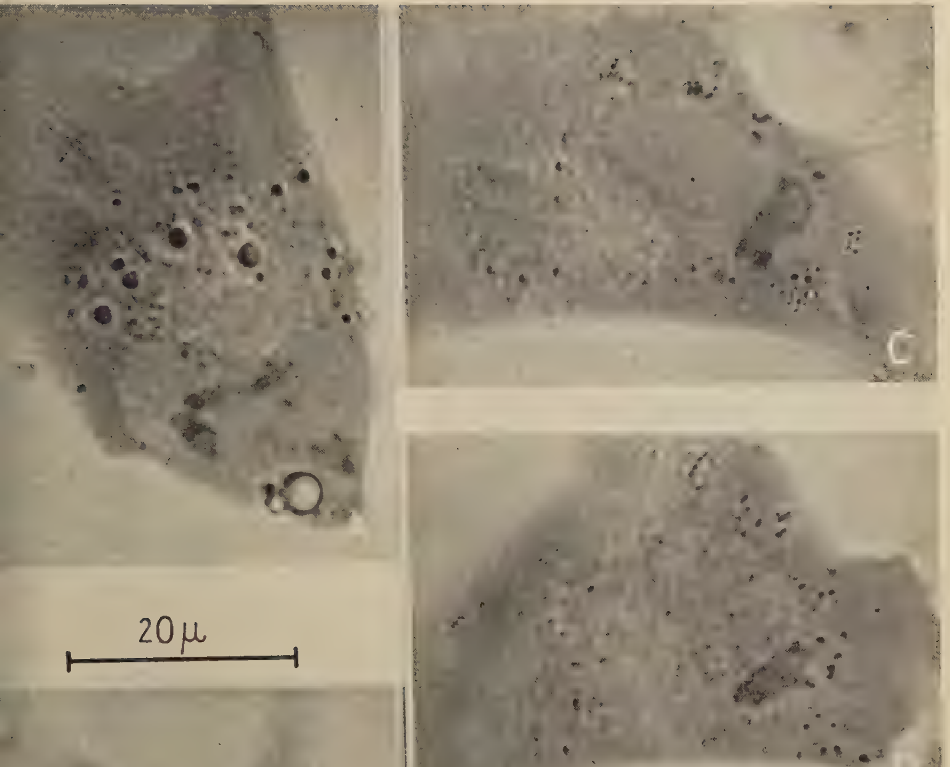
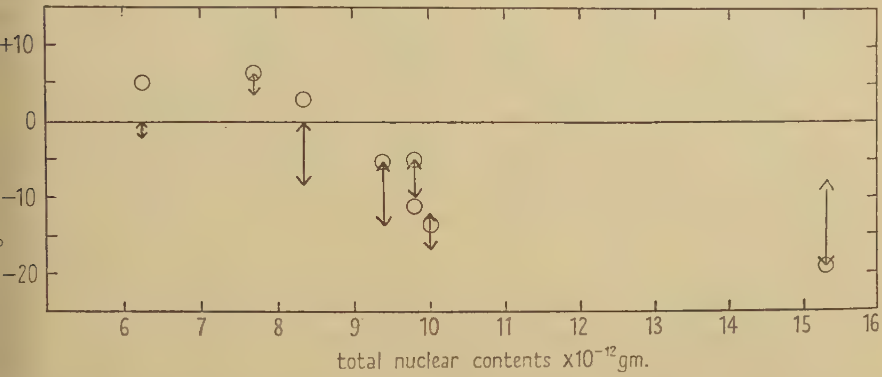


Fig. 15

H. G. DAVIES

th Tyrode. Immediately, and in a most striking manner, the nucleus verted to its homogeneous state (fig. 15, E).

In fig. 16 the changes in total nuclear extinction at 265 mμ are relative to e cell when first in Tyrode. With the appearance of refractile structures in e nucleus the non-specific light loss becomes measurable and the changes in tal extinction are indicated by the upper and lower arrows according to hether the non-specific loss varies as λ^0 or λ^{-4}. The small decrease in total



G. 16. Per cent. changes in total nuclear extinction at 265 mμ of the nucleus of the living ll during reversible changes brought about by altering the pH of the medium around the ll. Abscissae: total amount of absorbing material expressed as DNA. Ordinates: per cent. anges relative to the living cell when first in Tyrode (zero ordinate). The changes at pH 4·6 e shown by the upper and lower arrows when non-specific light loss is corrected according to and λ^{-4} respectively. The circles show the changes when the cells are re-immersed in yrode and the nucleus once again homogeneous; there is a relatively small residual non-specific light loss for which correction was made according to λ^{-2}.

tinction appears to increase in magnitude with the amount of material in e nucleus. After the cells have been re-flooded with Tyrode (circles) there hardly any change relative to the cell in acid medium.

DISCUSSION

We must first consider the possibility whether the changes observed after xation may be due in part to ultra-violet radiation damaging the cell. It was own (Davies, 1952) that the dosage required for one photomicrograph at 5 mμ in the apparatus used may disturb the metabolism of the cell and that e series of photomicrographs required for a complete absorption spectrum ould lead to eventual damage to the cell. However, Walker and Davies 951) showed that no change in the total nuclear extinction could be detected ter about fifteen photomicrographs at 265 mμ taken at intervals over a period f 3 minutes. In the present experiments the dosage was less and cells were xed immediately after irradiation. Also, the low value for E_{265}/E_{280} was bserved in fixed cells which had not been previously irradiated in the ultra-iolet. Hence it is concluded that the changes observed after fixation are not any way due to irradiation of the cells by ultra-violet light.

I. *Total amount of absorbing material in the nucleus*

(i) *Changes in total amount after fixation.* When chick fibroblasts are fix in formalin and Carnoy the total nuclear extinction at 265 mμ decreases. Th change might be due to an actual loss of absorbing substances but oth factors complicate this obvious interpretation.

(*a*) The optical extinction in the area occupied by the nucleus of the livi interphase cell is due partly to the nucleus itself and partly to the layers cytoplasm above and below it. Conceivably, shrinkage of the nucleus witho a corresponding cytoplasmic shrinkage might cause some decrease in the to extinction of the area occupied by the nucleus. It has so far proved impossib to measure the contribution made by the cytoplasm to the absorption of t nuclear area, but there are reasons, summarized by Walker and Yates (195 for believing that this contribution might be small. It seems likely that t changes in the total extinction of the nuclear area due to differential shrinka will be small.

For the sake of brevity, measurements of optical extinction in the ar occupied by the nucleus have been, and will be, referred to as measuremen of the nucleus. It is to be understood that an indeterminate fraction of t measured extinction, probably small, is due to over- and under-lying cyt plasm.

(*b*) The heterogeneity produced by fixing the cell might result in a decrea in the total nuclear extinction (Introduction (iii)). Theory indicates that t errors due to distribution are likely to be small compared with the chang actually observed since (1) the cells have a low optical extinction ($< 0{\cdot}2$), a (2) densities are recorded with an aperture that is small compared with t area of the nucleus. The experiments in which the pH of the medium arou the cells was varied may be interpreted to support this conclusion. Thus, the rapid reversal from the heterogeneous to the homogeneous state of t nucleus there is on the average a negligible change in the total extinction 265 mμ. Conceivably the first drop in total nuclear extinction, which is, in a case, less than that observed during fixation, is due to an actual loss of absor ing material during the long period, up to 1 hour, in acid medium. Als results obtained after freezing-substitution show that the distributional err is small. A few cells were measured, the heterogeneity of which was com parable with that of Carnoy-fixed cells. They showed no decrease in tot nuclear extinction at 265 mμ.

(*c*) The non-specific light loss due to the heterogeneity of the fixed cell all introduces some uncertainty in the results. However, in these fixed nuc E_{313}/E_{265} is never very high and is usually less than 15 per cent. Hence t changes in the total extinction of the nuclei of chick fibroblasts due to fixati are not seriously affected by the method used to correct for non-specific lig loss; the greatest uncertainty is in cells fixed with Carnoy's fluid.

In other types of cells (Caspersson, 1950, Nurnberger and others, 195 E_{313}/E_{265} is often much larger, ranging from 30 to 50 per cent. Conceivab this might be due partly to a higher ratio of light-scattering substanc roteins, &c.) to light-absorbing substances (nucleic acids, &c.). Much of the aterial is in the form of sections and it is possible that further treatment after xation increases non-specific light loss. It has been noted, for example, that ere is an unusually high optical extinction at 313 mμ in fibroblasts fixed by eezing-substitution and left overnight in the methyl alcohol. This has been ttributed to a hardening of the proteins by the alcohol, resulting in sharper radients of refractive index which increase light scatter (Caspersson, 1950).

(*d*) There is no way of assessing whether any of the observed changes are ue to failure of Beer's law. The magnitude of the glare was determined by hotographing an opaque object (Wilkins, 1950), the measured optical extincion of which was about 1·0 at 265 mμ. The procedure described by Davies nd Walker (1953) was used and it was concluded that there was a linear elationship between the measured and true optical extinctions over the range ncountered in this investigation. Hence no error was introduced by glare.

(*e*) It has been shown (Kunitz, 1950) that digestion of DNA with desoxyibonuclease causes an increase of about 30 per cent. in the optical extinction t 260 mμ; this increase generally precedes the liberation of free acid or he formation of acid soluble split products. Conceivably fixation also might hange the structure of the DNA and hence alter its absorption. This may be reason for the increase in the total nuclear extinction after freezing-substituion.

It has been concluded that the experimentally observed decreases in total uclear extinction ranging from about 10 to 35 per cent. after formalin and Carnoy fixation roughly represents the magnitude of an actual loss of absorbing substances from the nucleus. If there were an increase in absorption due o changes in the structure of the DNA on fixation, then the loss of absorbing substances would be greater than that indicated by this range of values. The approximate agreement between the values of total nuclear extinction before and after methanol freezing-substitution, corrected for light scatter according to λ^{-4}, is regarded as fortuitous.

(ii) *Nature of the absorbing substances in the nucleus.* The range of values obtained for the contents of the nuclei of living chick fibroblasts, expressed as DNA, is $5–15 . 10^{-12}$ gm., in agreement with the extensive data of Walker and Yates (1952). These workers have drawn attention to the fact that these values are considerably higher than the DNA content of diploid differentiated cells from the same species. Thus, using ultra-violet microspectrometry, they obtained $3{\cdot}0 . 10^{-12}$ gm. for the DNA content of the chick erythrocyte, in fairly good agreement with the value $2{\cdot}5 . 10^{-12}$ gm. obtained by bulk biochemical measurements (Davidson and Leslie, 1950). Walker and Yates have shown that some of the increase in ultra-violet-absorbing material in the interphase nucleus is due to the synthesis of DNA in preparation for the next mitosis. But as this would be expected to yield a range of values approximately $3–6 . 10^{-12}$ gm. it was concluded that absorbing substances other than DNA are present in the interphase nucleus. In view of these results several fixed nuclei were digested with ribonuclease and it was found that on average the total nuclear extinction was reduced by about 50 per cent.; the nucleoli we not included in the measurements. However, such a high value for th ribonucleic acid content of the interphase nucleus cannot be accepted with out more extensive investigation.

We can at present only speculate as to the nature of the material lost o fixation with formalin and Carnoy. It is likely that unbound molecules of lo molecular weight originally present in the nucleus, or produced by fixatio would diffuse away during fixation. Indeed Walker and others (1952) hav suggested that the substances lost during fixation are nucleotides of low mole cular weight which are precursors of DNA. It is interesting that these sma molecules, if they are present, do not diffuse away during freezing-substitu tion. Clearly chemical analyses of the fixing fluids are needed to determine th precise nature of any substance lost by fixation. This would require the use much larger numbers of cells than were available in this investigation.

II. *The absorption spectra of the nucleus*

(i) *The living nucleus.* It will be recalled that E_{260}/E_{280} for isolated DNA approximately 1·83 and that when protein is present the ratio decreases. O this basis it was concluded that the amount of protein in the nuclei of thes living cells is small compared with the amount of nucleic acid. However, th absorption spectrum of the nucleus appears to differ from that of isolate DNA since (1) E_{265}/E_{280} is often greater than 1·83, and (2) there are bump on the spectra in the region 270–280 mμ. A typical spectrum for an air-drie film of nucleic acid was obtained when calibrating the apparatus (Walker an Davies, 1950); E_{265}/E_{280} was approximately 1·7.

Results (Davies, Wilkins, Chayen, La Cour, 1954) for the total dry mass of th living nucleus obtained by interferometry do not agree with those obtained b microspectrometry, which shows a relatively low protein content. The metho makes use of the fact that the mass of material other than water in a cell ma be obtained from the optical path difference of the cell, a measurement whic is conveniently made by interference microscopy. The dry masses obtaine for the nuclei of several living fibroblasts were about $30 . 10^{-12}$ gm. and thi is to be compared with the total contents absorbing at 265 mμ ranging fro 5 to $15 . 10^{-12}$ gm. Hence it would be concluded from these combine measurements that the amount of protein is several times greater than th total amount of absorbing material.

(ii) *The fixed nucleus.* One change in the absorption spectra of the nucle common to all the fixatives employed is the marked drop in E_{265}/E_{280} (table from about 2 to 1·3, a decrease outside the limits of experimental error. Fro the equations of Caspersson and Santesson (1942) it appears that in contras to the relatively lower amount of 'standard protein' in the living nucleus, th amount of protein in the fixed nucleus is about an order of magnitude greate than the amount of nucleic acid. Some comments will be made on the factor which might conceivably change the shape of the absorption spectrum an hence produce this result.

(*a*) The errors in microspectrometry are usually a function of optical extinc- on and, since shrinkage causes the extinctions of the living and fixed cells differ, often widely, this might cause a change in shape of the absorption ectrum in the manner observed. For the reasons stated in the Discussion, (i), however, these errors are small in these cells and hence will not produce e marked change observed. Any loss of substances absorbing like DNA, fter formalin and Carnoy fixation, would be expected to change E_{265} and $_{280}$ in such a manner as to leave the ratio unchanged. After freezing-sub- itution, when there is no decrease in total nuclear absorption at 265 mμ, $_{265}/E_{280}$ decreases to a similar value.

(*b*) Conceivably, substances with a relatively high optical extinction at 80 mμ might diffuse into the nucleus from the cytoplasm. However, a imilar decrease in E_{265}/E_{280} was recorded in the cytoplasm of cells fixed by reezing-substitution (table 1).

(*c*) The ratio E_{265}/E_{280} is unaffected by the way in which the correction is ade for non-specific light loss. However, changes in light scatter resulting rom changes in the refractive indices of the substances in the cell with wave- ength have previously been ignored. If the dispersion in the region of the ucleic acid absorption band were anomalous, the refractive index of the cell nd, hence, the light scatter, might be relatively greater at 280 than at 265 mμ. his would result in a relative increase in optical extinction at 280 mμ which ould cause a decrease in the ratio E_{265}/E_{280}. Measurements of the refractive ndices of nucleic acids and proteins at absorbing wavelength are required to est this possibility, which, however, seems an unlikely one owing to the low alue of the non-specific light loss measured by the optical extinction at 13 mμ.

(*d*) The method of curve analysis is based on the supposition that the bsorption spectrum of nucleic acid and protein in combination in the cell an be obtained by simple addition of the absorption spectra of the individual ubstances in solution outside the cell. However, the absorption spectrum of ne substance is often altered by combination with another or by changes in he degree of polymerization, &c. Hence different spectral shapes may be roduced by, and might eventually be used to elucidate, differences in the tate of nucleic acids and proteins in cells. The results suggest that the erivation of relative amounts of nucleic acid and protein from absorption pectra of the living or the fixed cell must be viewed with caution.

III. *Comparison of fixatives*

Although fixation with osmium tetroxide results in little change in cell structure, it is unsuitable for quantitative measurements due to the staining by the fixative. Likewise, formalin is contra-indicated since it results in large losses of cytoplasmic absorption. Freezing-drying has long been suggested as the most suitable method of preserving cells for ultra-violet microspectro- metry (Caspersson, 1940). This investigation shows that after freezing-drying, as after freezing-substitution, (1) the appearance of the cell is very lifelike,

(2) the material in the cell may be preserved in a nearly homogeneous state an hence the light scatter and distributional errors are at a minimum, and (3) th absorption spectrum of the nucleus is altered. Shrinkage after freeze-substitution will affect the measurements of concentration but not of total amount of substances in the cell. After freezing-drying, cells have a low non-specifi light loss as a result of swelling of the cell proteins in the doubly distille glycerol in which they are mounted; this eliminates sharp changes in refractive index (Caspersson, 1940). Conceivably, proteins precipitated wit alcohol will be less likely to swell in glycerol and hence it is likely that, i general, non-specific light loss will be greater in cells fixed by freezing substitution. Measurements have yet to be made to see if freezing-dryin preserves total nuclear extinction.

Freezing-substitution has the advantage that it is a much quicker metho than freezing-drying. Also, cells after freezing-substitution are immediatel available for further measurements in aqueous media, for example, afte enzyme digestion. It seems likely that for such measurements there will b little to choose between a tissue culture cell fixed by alcohol freezing substitution and a frozen-dried cell fixed by alcohol.

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TABLE 1

Fig. no.	*Fixation*	*Region of cell*	*Living* E_{265}/E_{280}	*Fixed*		
				E_{265}/E_{280} *uncorr.*	E_{265}/E_{280} *corr.* λ^0	E_{265}/E_{280} *corr.* λ^{-4}
3, A and B	Formalin	Nucleus	1·65	1·21	1·29	1·23
. .	”	”	2·20	1·17	1·17	1·16
5, A and B	Carnoy	”	1·85	1·23	1·26	1·22
7 A	Osmium	”	2·2	. .	. .	. .
10, A and C	Freezing-substitution	”	2·0	1·46	1·50	1·49
10, B and D	”	”	2·18	1·43	1·47	1·46
. .	”	Cytoplasm	1·84	1·30	1·34	1·32
14	Freezing-drying	Nucleus	. .	1·46	1·46	1·46

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The Effect of Diet on Egg Maturation and Resorption in *Mormoniella vitripennis* (Hymenoptera, Pteromalidae)

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SUMMARY

1. *M. vitripennis* females have been treated in one of the following ways: (*a*) fed on honey, (*b*) starved, (*c*) fed on host blood.

2. When fed on honey the ovaries contain 22 eggs after 2 days, then a slow cycle of maturation and resorption begins so that for 16 days their condition does not change. After 16 days resorption is more rapid and by 28 days there are only 1 or 2 mature eggs.

3. When starved, the parasites die in 5 days. Rapid resorption occurs and at death there are only 3 eggs in the ovaries.

4. When fed on host blood, eggs mature rapidly. After 5 days the ovaries contain 40 mature eggs even though 260 have been deposited. Parasites which are then starved, resorb eggs very rapidly and die in 48 hours, but those fed on honey live for at least 8 days and rapid resorption does not occur.

INTRODUCTION

MORMONIELLA VITRIPENNIS (Walker) is parasitic on the pupal stage of Muscoid flies. The female drills through the host puparium and lays a number of eggs on the enclosed pupa. The eggs hatch into larvae which feed on the host tissues and pupate inside the host puparium from which the resulting adult parasites eventually bite their way out. After ovipositing, the female parasite forms a feeding tube and sips up some of the host's blood. This blood meal is not essential for the maintenance of life, as the parasites will live for several weeks on a diet of honey, but it has a marked effect on the condition of the ovaries.

Roubaud (1917) records that when *M. vitripennis* females are fed on glucose only, they have 4–5 well-developed eggs in the ovaries after 12 days, but when fed on host blood, they have ovaries 'bulging with eggs' after 4–5 days. It was further shown by Flanders (1935) that when deprived of hosts, many parasitic Hymenoptera resorb the mature eggs present in the ovaries. The phenomenon has been recorded in several species, but the only quantitative work appears to be that of Grosch (1950), who counted the number of eggs in the ovaries of *Habrobracon juglandis* (Ashmead) at various stages of starvation.

The present quantitative work on the maturation and resorption of eggs in *M. vitripennis* was undertaken as a preliminary to a behaviour study. It is obvious that the behaviour pattern will be affected by the condition of the ovaries and it is therefore important to know what changes occur with increased age and with different diets. It is also advantageous if a method of

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rearing can be devised such that the condition of the ovaries at any time can be predicted without killing the parasite and dissecting it.

Standardization of material

By observing parasitized puparia in transmitted light it can be shown that *M. vitripennis* imagines spend 20–30 hours inside the host puparium before biting their way out, so that an error is introduced if the age of the imago is measured from the time it leaves the host. To avoid this error host puparia were opened when the parasites were still in the pupal stage, 48 hours before eclosion. The ages given are accurate to ±2 hours.

As the overall length of the imago varies not only with the contents of the gut and ovaries, but also through muscular action, the parasites were measured in the late pupal stage, and only those having a length of 2·00 mm. ±0·05 mm. were chosen. The resultant imagines were about 2·5 mm. long, and each was confined with a male immediately. Scrutiny of the spermatheca after the experiment showed that in every case mating had taken place. The parasites were reared, and all experiments were carried out at a temperature of 25° C. and relative humidity 75 per cent.

Puparia of the house fly *Musca domestica* L. were used as hosts. The fly larvae were reared on milk at a temperature of 25° C. and were kept at that temperature until required for experiments. They were used 24 hours after the onset of pupation, and measured 6·00±0·2 mm. in length.

Method of recording

For ease of comparison a method of recording was devised whereby the condition of the ovaries could be expressed as a series of numbers indicating the number of eggs at five different stages of maturation and resorption. The ovarioles are polytrophic and the greater part of them is packed with numerous minute oocytes measuring less than 0·1 mm. in length, accompanied by a slightly larger group of nurse cells. These oocytes have not been recorded, partly because counting them would be extremely laborious, but mainly because there was never any obvious change in their number throughout the experiments.

The freshly killed insects were dissected and the ovaries removed in a drop of saline. The early stages of maturation and the late stages of resorption show up plainly, but as the transitional stages are poorly defined the ovaries were stained for 1 minute in acetocarmine. Mature eggs take up no stain, but immature and resorbing eggs stain deeply.

Immature eggs were divided into two groups, namely, 'three-quarter-mature eggs' in which the ratio between the length of the oocyte and its group of nurse cells was greater than 3, and 'half-mature eggs' in which this ratio lay between 1 and 3. If this ratio was less than 1, no record was made. Resorbing eggs were also divided into two groups—those which were in the early stages of resorption, as shown by their shape and size, and those in later stages which had become smaller and in which the oval outline had been lost.

The number of eggs falling into each category was written down in order, e half-mature eggs first, and the mature eggs underlined for extra clarity. s an example the ovary shown in fig. 1, which has 6 half-mature eggs, 7 ree-quarter-mature eggs, 24 mature eggs, 2 in the early stages of resorption d 2 in the late stages, would be recorded as 6–7–*24*–2–2. This is clearly not accurate method of recording, but it is quick and gives a good general icture of the condition.

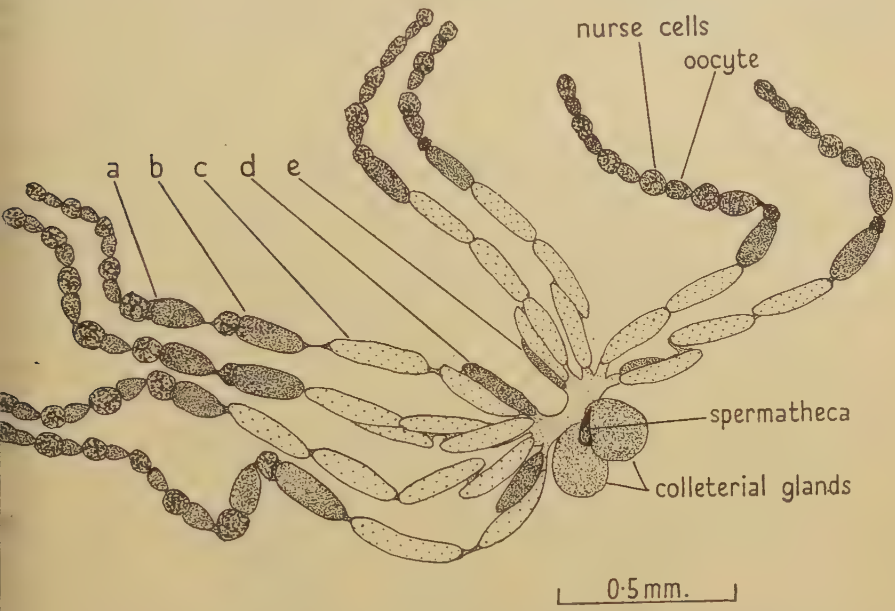


FIG. 1. The ovaries of *M. vitripennis* showing eggs at various stages of maturation and resorption. The distal part of each ovariole has been omitted (semi-diagrammatic).

a = Half-mature egg.
b = Three-quarter-mature egg.
c = Mature egg.
d = Egg in early stage of resorption.
e = Egg at late stage of resorption.

The subdivisions were chosen arbitrarily before the work started and it was subsequently apparent that the difference between a 'half-mature egg' and a 'three-quarter-mature egg' was greater than that between a 'three-quarter-mature egg' and a mature one. When eggs are maturing rapidly the difference in time between a 'three-quarter-mature egg' and a mature egg is probably less than 1 hour, and a better estimate of the number of eggs available for oviposition is obtained if these two categories are lumped together. This has been done when calculating the egg maturation rates.

The ovaries of parasites fed on honey only

The condition of the ovaries of standard females deprived of hosts and fed only on honey, is shown in table 1. The first eggs mature when the imago is between 1 and 2 days old. Resorption begins at about 3 days and continues indefinitely. Eggs continue to mature at a low rate and there appears to be a slow cycle of maturation and resorption, the two processes just keeping pac with each other for the first 16 days so that the general condition of th ovaries remains more or less constant. The change in maturation rate durin the first 3 days can be obtained from table 1 by observing the increase in th number of mature and three-quarter-mature eggs on consecutive days. Th rates for the first 3 days are 9, 13, and 3 respectively. It is not possible to obtai an accurate estimate for longer than 3 days because there is no way of knowin how many eggs have been resorbed, but the rate is clearly not more than 1– per day (see fig. 2).

TABLE 1

The condition of the ovaries of standard-sized M. vitripennis *females deprived o hosts and fed only on honey*

Age in days	*Sample size*	*Mean condition of ovaries* *a b c d e*
0	4	0–0– *0*–0–0
1	13	8–9– *0*–0–0
2	31	7–4–*18*–0–0
3	6	7–1–*24*–1–0
4	6	5–0–*20*–1–2
5	6	4–1–*19*–0–1
6	6	2–2–*19*–2–2
7	7	2–0–*16*–1–2
8	7	3–2–*15*–1–1
9	6	3–1–*14*–0–2
10	12	5–1–*16*–2–1
12	7	4–2–*15*–2–1
16	6	2–1–*15*–3–2
20	3	3–1– *5*–2–3
22	1	1–1– *2*–0–2
28	2	1–0– *1*–0–3

a = No. of half-mature eggs.
b = No. of three-quarter-mature eggs.
c = Mature eggs.
d = Eggs in early stage of resorption.
e = Eggs in late stage of resorption.

The importance of this to the parasite is that at any time during that period a fairly large number of freshly matured eggs is always available. The numbe of immature eggs is small, however, so that once the supply of mature eggs ha been exhausted, there will be an interval before more eggs are ready. Afte 16 days the condition of the ovaries rapidly deteriorates and by the end o 28 days the condition has become 1–0–*1*–0–3.

A noteworthy point is the extreme constancy of the ovaries of females aged 48 hours. In a batch of 31 females the mean number of mature and three-quarter-mature eggs together was 22 with a standard deviation of only 2·05. This fact has been utilized in the later experiments where it was desirable t know the number of eggs in the ovaries without dissecting the female.

e ovaries of starved parasites

If females are starved from eclosion the shortage of immature eggs is parent at 48 hours, when the condition of the ovaries is 1–0–*16*–0–0. Resorp n begins at 48–72 hours and by 5 days, a few hours before death, the con- ion has become 2–0–*3*–8–2. The situation in *M. vitripennis* appears to differ om that found by Grosch (1950) in *Habrobracon juglandis*, for he records at the mature eggs were resorbed by the sixth day, the immature ones by the nth day, and death followed on the tenth day.

ie ovaries of small parasites

The importance of standardizing the size of the females was shown by nsidering the ovaries of a batch of females which measured only 1·6 mm. as ipae. Table 2 shows that, although the general trend is similar, the ovaries ever contain as many eggs as those of the larger females. This agrees with the ndings of Jacobi (1939), who showed that the reproductive power of small *. vitripennis* females is less than that of large ones.

TABLE 2

he condition of the ovaries of 'small' M. vitripennis *females deprived of hosts and fed only on honey*

Age in days	*Sample size*	*Mean condition of ovaries* *a b c d e*
0	2	0–0–*0*–0–0
1	4	2–2–*1*–0–0
2	2	2–3–*2*–0–0
3	4	1–1–*8*–0–0
5	6	1–0–*3*–0–0
7	2	2–0–*3*–1–1
8	2	1–1–*4*–0–2
12	4	1–1–*3*–0–1
16	4	0–1–*3*–1–1
20	3	0–0–*2*–0–1
24	4	0–0–*1*–0–1
30	1	0–0–*0*–0–0

a = No. of half-mature eggs. *b* = No. of three-quarter-mature eggs.
c = No. of mature eggs. *d* = No. of eggs in early stage of resorption.
e = No. of eggs in late stage of resorption.

'he ovaries of parasites fed on host blood

For these experiments newly eclosed females were first fed on honey for 8 hours, and then confined in a 3-inch by 1-inch tube with an ample supply f puparia for periods of between 1 and 5 days. Females of this type were hosen because (1) the condition of the ovaries could be predicted (as shown bove), and (2) parasites less than 36 hours old display little interest in puparia. After the experiments the females were immediately dissected, bu the puparia were kept at 25° C. until the unparasitized hosts had emerge The remaining hosts were then opened to discover whether they had bee stung by the parasite or had died from natural causes. If the host had bee stung, the number of parasite progeny was counted and recorded. There no polyembryony in *M. vitripennis*, and as a test batch of fifty hosts showe that egg mortality is negligible, the number of progeny can be considere equal to the number of eggs laid.

TABLE 3

The conditions of the ovaries of standard-sized M. vitripennis *females which were fed on honey only for the first 48 hours after eclosion and then given host blood for the number of days shown.*

Age in days	*Duration of blood meal in days*	*Sample size*	*Mean condition of ovaries* *a b c d e*	*Mean number of eggs laid during period*
3	1	14	12– 8– *8*–0–0	21
4	2	14	13–12–*10*–0–0	50
5	3	3	16–22– *8*–0–0	144
6	4	3	10–20–*13*–0–0	199
7	5	3	15–19–*19*–0–0	250

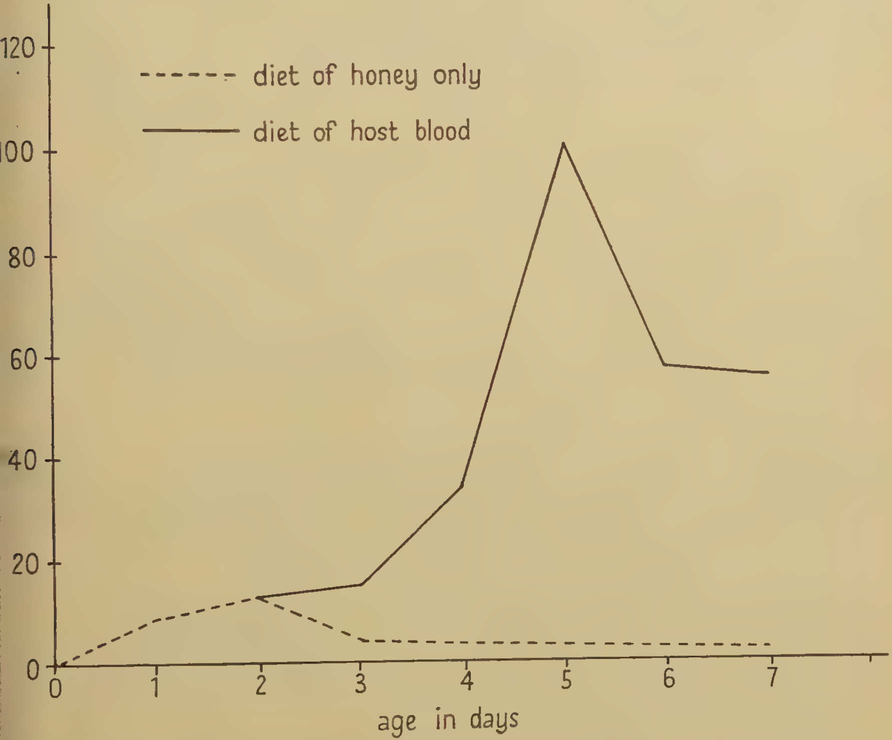
a = No. of half-mature eggs.
b = No. of three-quarter-mature eggs.
c = No. of mature eggs.
d = No. of eggs in early stage of resorption.
e = No. of eggs in late stage of resorption.

The average condition of the ovaries at the end of the experiment is show in table 3. The most noticeable difference between these and the ovaries females fed on honey only is in the large number of immature eggs presen The number of mature eggs is, of course, not large as the parasites have bee ovipositing continuously. The mean number of eggs laid during the experiments is given in column 5. Although the samples were small, an estimate the maturation rates can be obtained by adding the number of eggs la during the experiment to the number in the ovaries after the experiment, an subtracting 22—the number in the ovaries at the start of the experimen Mature eggs and three-quarter-mature eggs are lumped together here for th reason given previously. From these figures, which give the number of egg maturing over the whole period, the daily rate can be obtained. This is a bett method than giving the parasites a fresh batch of hosts every day and countin the number of eggs laid, partly because of the danger of disturbing th parasite and partly because it would not be possible to take into account th number of eggs still in the ovaries at the end of each day.

The mean daily estimates are shown graphically in fig. 2. It is seen th when the parasites are fed on host blood the maturation rate rises to a peak

2 on the third day and then drops to little more than half that figure on the o following days. In the same way the mean daily number of hosts stung the parasites can be estimated, and it is found that these figures also rise pidly from 2·4 on the first day, to a peak of 12·2 on the third day, dropping ain to 9·0 by the fifth day.

These results are in broad agreement with those of previous authors. nirnov and Kuzina (1933) record that one *M. vitripennis* female stung 98 *illiphora* puparia in 12 days, and produced 745 offspring—a mean oviposition



IG. 2. The effect of diet on the egg maturation rate of *M. vitripennis*; a comparison between e daily egg maturation rates of females which have been (*a*) fed on honey only, and (*b*) allowed to feed on host blood after a diet of honey for 2 days.

ate of over 60 per day. Van der Merwe (1943), using *Lucilia* puparia as hosts, ives the mean oviposition rate as 50 per day. Cousin (1933), who gives the verage as only 15–17 per day, limited the parasites to two *Lucilia* puparia aily. None of these workers appears to have standardized the size of the emales.

he effect of host scarcity

A situation likely to arise in nature is that in which a parasite finds a number f hosts, oviposits, and feeds, and is then unable to find more for several days.

It is interesting to consider what happens to the ovaries under these circu
stances. Newly eclosed females were fed on honey for 48 hours, given amp
hosts for the next 48 hours, and then starved. No female survived more th
48 hours' starvation and dissections showed that resorption was proceedi
very rapidly whilst few eggs were maturing (see table 4).

TABLE 4

The condition of the ovaries of standard-sized M. vitripennis *females which h
been given honey for the first 48 hours after eclosion, host blood for the next
hours, and finally starved.*

Age in days	*Period of starvation in hours*	*Sample size*	*Mean condition of ovaries* *a b c d e*
4·0	0	14	13–12–10– 0–0
4·7	16	2	3– 3–25– 7–1
5·0	24	5	8– 0–34– 3–1
5·7	40	7	3– 0– 5–10–4

a = No. of half-mature eggs.
b = No. of three-quarter-mature eggs.
c = No. of mature eggs.
d = No. of eggs in early stage of resorptio
e = No. of eggs in late stage of resorption.

TABLE 5

The condition of the ovaries of standard-sized M. vitripennis *females which h
been given honey for the first 48 hours after eclosion, host blood for the next
hours, and then returned to a diet of honey*

Age in days	*Duration of second honey-meal in days*	*Sample size*	*Mean condition of ovaries* *a b c d e*
4·0	0	14	13–12–10–0–0
4·7	0·7	3	4– 5–33–3–0
5·0	1·0	3	3– 1–46–0–1
6·0	2·0	4	5– 1–39–1–2
8·0	4·0	2	3– 1–40–3–1
9·0	5·0	2	2– 1–37–3–2
12·0	8·0	2	2– 1–23–1–2

a = No. of half-mature eggs.
b = No. of three-quarter-mature eggs.
c = No. of mature eggs.
d = No. of eggs in early stage of resorptio
e = No. of eggs in late stage of resorption.

The rapid death of the females under these circumstances as compared wit
their survival for 5 days when starved from eclosion is presumably linked wit
the size of the fat-body. Newly eclosed females have a large reserve of f
which disappears after 4–5 days.

In nature the parasites might be expected to feed on nectar or honey-de hosts were scarce, and the effect of this can be demonstrated by providing em with honey. Standard-sized females after being fed on honey for 48 urs were given ample hosts for 48 hours and then returned to a diet of ney only. They lived for at least 8 days—the experiments were not conued longer—and during most of that time the number of mature eggs did t fall greatly. A slow cycle of maturation and resorption occurred and the neral condition of the ovaries was little changed at least for the first 5 days ee table 5).

It appears that under starvation conditions the eggs are resorbed and their ntents used in general body metabolism. If honey is available for this general etabolism the parasites can live for a considerable period and maintain a rge number of eggs in the ovaries. The importance of this to a parasite whose ost is found in isolated groups is obvious.

Discussion

The method of rearing has a very great effect on the condition of the ovaries f *M. vitripennis* and this in turn will affect the behaviour pattern. A detailed orrelation between the ovary condition and the behaviour pattern has not een carried out, but it has been observed that females which have been eprived of hosts for 5 days are unable to parasitize more than 1 or 2 hosts n the next 36 hours whereas those which have had ample hosts parasitize 12–5 in the same time. Presumably after the mature eggs in the ovaries have een deposited there is a delay whilst others are maturing.

If a female *M. vitripennis* is dissected soon after feeding on host blood the nid-gut is distended with a pale straw-coloured fluid. As digestion proceeds he colour gradually darkens until it becomes black at the end of 48 hours. f fed on pure sucrose the crop becomes distended whilst the mid-gut remains flaccid and does not darken. An interpretation of this could be that arbohydrates are digested in the crop, but host blood, and probably all proteins, are digested in the mid-gut. Females fed on honey showed a slight discoloration of the mid-gut after 3 weeks. This does not occur when females are fed on pure sucrose, and may indicate that the small amount of protein in the honey was being used for egg production. This could be checked by making a comparison with the condition of the ovaries of females which had been fed on pure sucrose only. Under natural conditions, however, females feeding on nectar or honey-dew would also take in small quantities of protein which might have the same effect as the protein in honey.

An egg which has been resorbed leaves no trace in the ovariole, so that the number of eggs being resorbed at any one time bears no relation to the number of eggs already resorbed. Flanders (1942*a*) states that in *Encyrtus fuliginosus* the complete exochorion is extruded into the body cavity and that 10–100 of these may be found free in the body fluids. During the present work no trace of exochorions of resorbed eggs could be found in the body fluid. As the exochorion in this species is smooth and transparent it may be difficult to find; on the other hand, careful scrutiny of stained preparations of ovari shows that the egg loses its shape and the chorion apparently disappears at early stage of resorption.

Flanders (1942*b*) found that some non-viable eggs are laid when a parasi has been deprived of hosts. He suggests that these are eggs in the early stag of resorption in which the chorion is still tough enough to withstand th passage through the ovipositor. Partly deflated eggs of *M. vitripennis* ha occasionally been found on a host, but it has been impossible to decide wheth these were in the early stage of resorption, or whether they had been damage during oviposition.

No attempt has been made to determine the exact process by which eg resorption occurs. The contents are not taken back by the nurse cells, as the have disappeared; it is not initiated by special cells in the wall of the ovario because often a mature and a resorbing egg are found lying side by side; seems likely that a change in the structure of the egg starts the process. N attempt has been made to discover how this is affected by the general con dition of the insect, but work along these lines is continuing.

Most of this work was carried out in the Hope Department of Entomolog Oxford. I wish to express my thanks to Professor G. C. Varley, who supervise it, and to Dr. B. M. Hobby for his many helpful suggestions.

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A Micro-injection System

By J. WORST

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SUMMARY

The apparatus works by the thermal expansion of water. The water is contained in fine, thick-walled capillary tube, one end of which is made into a micro-pipette. The ther extremity, which is closed, is heated over varying lengths by warm water in rder to obtain the required change in the volume of the water by thermal expansion.

MICRO-INJECTION, as it is used at present, is a modification of the ordinary procedure of injection, in which a reservoir connected to a icro-pipette is used. The necessary increase in pressure is effected by a ecrease in capacity (Chambers and Kopac, 1950). In all the current methods he ratio between the capacity of the reservoir and the micro-pipette is very arge, and this entails difficulties in their operation. Pierre de Fonbrune (1935) as come nearest to the ideal small-capacity micro-injector. His *injecteur hermique* consists of a short tube completely filled with water. Micro-injecions are made by heating the tube electrically so that the water expands. Unortunately, external temperature-changes strongly affect the system.

The system in this paper is based on the thermal principle of de Fonbrune. ig. 1 represents a de Fonbrune micromanipulator, on which a 'thermal njector' has been mounted. The tip of the latter extends into the oil chamber n which micro-operations are performed. The injector is essentially a bent, hick-walled capillary tube. The tail of the tube is heated by immersion in a est-tube of warm water. The simplest way to do this is to slide the test-tube n a slanting support, but the same effect can be obtained by changing the evel of the water by means of a syringe. The syringe is mounted on a support n the floor and is operated by rotating a circular foot-rest, the piston being noved in one direction by a pulley worked by the operator's feet, and in the ther direction by a spring (fig. 2). The heater, which is immersed in the testube to keep the water at a fairly constant temperature, is not represented in ig. 1.

The method of making the injector is illustrated in fig. 3.

1. A capillary tube is drawn from a thick-walled glass tube and filled with boiled distilled water (A in fig. 3).
2. The tube is sealed at one end in a small gas flame; at the other end a little water is boiled out (B), after which this extremity is also sealed (C).
3. The tube is heated locally, so that the vapour pressure blows a bubble (D).

[Quarterly Journal of Microscopical Science, Vol. 95, part 4, pp. 469–472, Dec. 1954.]

4. The bubble is drawn out (E).
5. The thin-walled section is made into a hook, and a bend is made in th thick-walled shaft (F).
6. The whole capillary is refilled by dipping the hook in water and heating the tail.

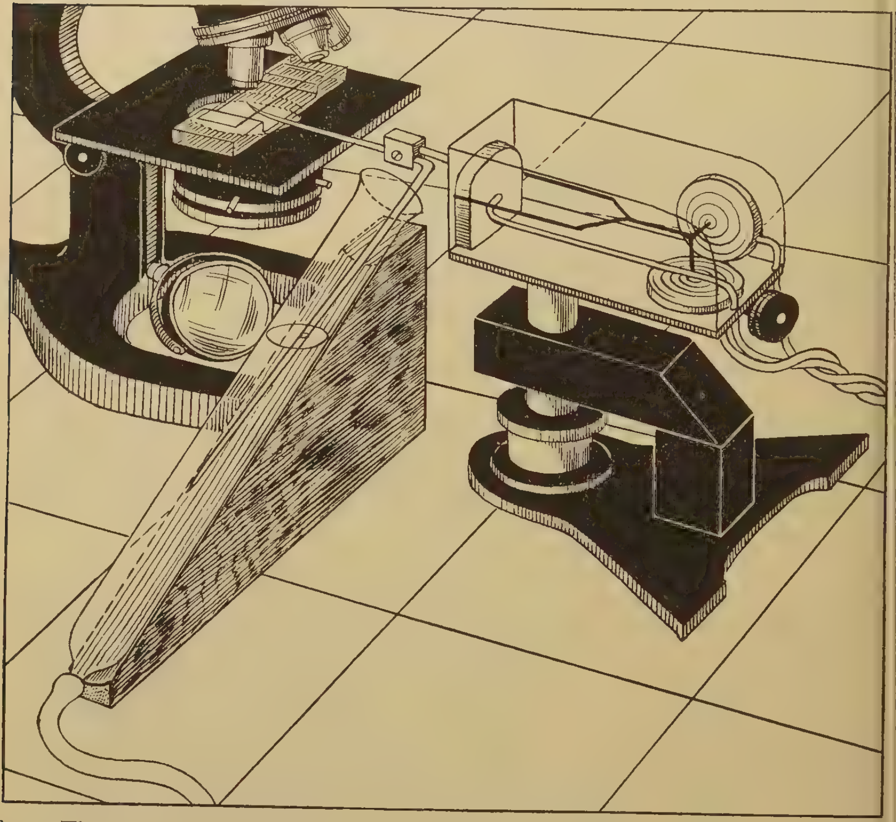

FIG. 1. The micro-manipulator, injector, and test-tube, in position in front of the microscope.

7. The micro-tip proper is made on the de Fonbrune micro-forge (de Fonbrune, 1950), the pulling force being provided by a small weight attached to the hook (G, H; the filament and capillary are shown in the field of the forge). The tube is drawn out, the finished micro-pipette containing a small air gap (I).
8. The air-bubble is replaced by paraffin oil after mounting on the micro-manipulator.

This micro-injecting system has the following properties:

1. The capacity is small.
2. The system offers possibilities for quantitative injections, as there are no air gaps and the amount of expanding water is directly proportional to the length of the tail heated and to the rise in temperature.

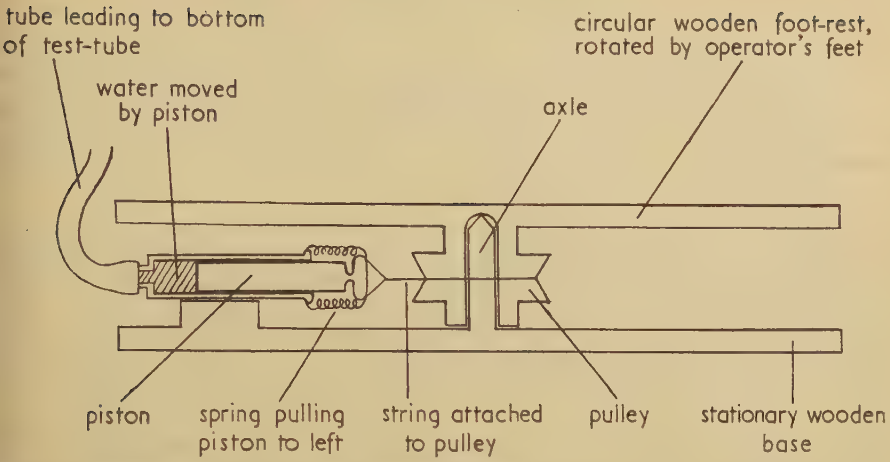
tube leading to bottom of test-tube
water moved by piston
axle
circular wooden foot-rest, rotated by operator's feet
piston
spring pulling piston to left
string attached to pulley
pulley
stationary wooden base

FIG. 2. The apparatus by which the level of the water in the test-tube is controlled (diagrammatic vertical section).

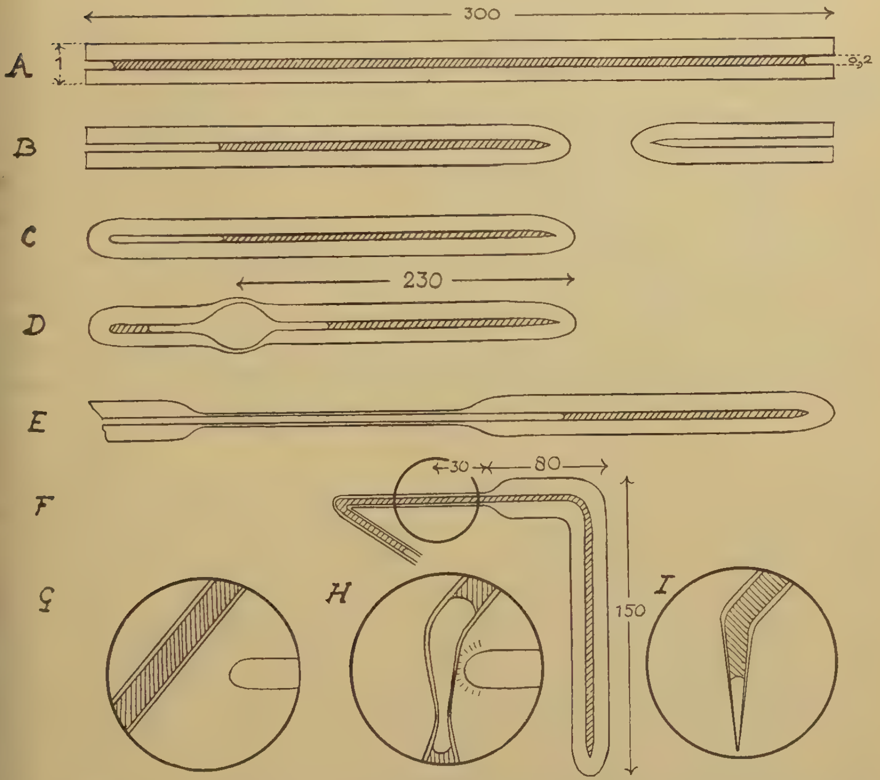

FIG. 3. The diagrams illustrate the method by which the injector is made. For description see text. The numerals represent measurements in millimetres.

3. The force of the expanding water partially eliminates the trouble o
clogging tips.
4. Pressure-control is accurate and the pressure-gradient is favourable.
5. The thick wall serves as a thermal insulator for the water in the capillary
Thus external temperature-oscillations are decreased and the pipett
'stays put'.
6. The response is immediate.

Besides injecting, the system is equally suitable for micro-aspirations such as slow draining, the speed of drainage being regulated by controlling the speed of cooling. It should also be useful in picking up single bacteria, as the water never rushes back into the pipette. Finally, as the injection pressure is high, injections could be made into rigid-walled plant-cells.

I am much indebted to Professor J. F. Danielli, Dr. M. J. Ord, Dr. L. G. E. Bell, and Dr. J. G. Thomas, all of the Department of Zoology, King's College, London, without whose constant encouragement I should not have been able to put their laboratory facilities to full use.

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On the Structure and Adaptations of *Pandora inaequivalvis* and *P. pinna*

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(*From the Department of Zoology, The University, Glasgow*)

SUMMARY

The two British species of *Pandora* are adapted for life on muddy-sand. *P. inaequivalvis* is taken from very sheltered shores at low-water mark and down to 10 fathoms. *P. pinna* is taken from depths of over 40 fathoms.

Both species are markedly inequivalve. This is probably connected with the habit of lying horizontally.

The mantle muscles and the ciliary mechanisms of the mantle and gills are described.

An account of the morphology and the functioning of the stomach is given and various modifications, due to the reduced typhlosole, are described.

PANDORA is the sole genus of the family Pandoridae (Eulamellibranchia) of the sub-order Anomalodesmata (Theile, 1935). There are two British species, *P. inaequivalvis* (Linné) and *P. pinna* (Montagu), but neither is common in British waters. Deshayes (1848) and Pelseneer (1911) have described the anatomy of *P. elongata* but, apart from observations by Ridewood (1903) and Atkins (1937*a*) on gill structure, there has been no study of living specimens. Observations have been made, therefore, on the structure and ciliary mechanisms of *Pandora* and comparisons have been made with species of related families, particularly the Lyonsiidae (Yonge, 1952) and Thraciidae (personal observations). Unless otherwise stated these observations apply to both the species *P. inaequivalvis* and *P. pinna*.

HABITAT

P. inaequivalvis is occasionally taken off Plymouth and Port Erin from muddy-sand in shallow water. The latter locality is apparently the northernmost record for this species. It is abundant at low water on sheltered sandy beaches in the Channel Isles and extends to the Mediterranean. Living specimens were obtained from Penpoull, a very sheltered muddy-sand beach near Roscoff on the Brittany coast. This beach shelved very slightly and much of it is used for oyster beds. The specimens of *Pandora* from this locality were not buried but lay at the surface on their sides. Although the animals were never seen to burrow, many had tufts of *Enteromorpha* and other algae attached to the siphonal end of the shell indicating possibly that they were often buried with only this part of the shell above the substratum.

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P. pinna has a greater geographical range than *P. inaequivalvis* and extend from Scandinavian waters to the Mediterranean. It is found in deeper wate than *P. inaequivalvis* and, whereas the latter is taken rarely from depth greater than 10 fathoms, *P. pinna* is always taken from 40 fathoms and below Specimens of this species were obtained from the Sound of Jura from muddy sand at a depth of 60 fathoms.

MANTLE/SHELL

Both species of *Pandora* are inequivalve with the right valve flat or even slightly concave (fig. 1). Yonge (1946) pointed out that, apart from *Aloidis* *Pandora* appears to be the only lamellibranch in which an inequivalve shell i not associated with horizontal disposition. However, the observations a Penpoull suggest that at times *P. inaequivalvis* does lie horizontally. Usually but not always, it was the right side that was found to be uppermost. The illustrations and description given by Forbes and Hanley (1853) show clearly the differences in shell shape between the two species. The maximum recorde measurements were: *P. inaequivalvis*, length 3·5 cm., height 1·9 cm., width 0·6 cm.; *P. pinna*, length 1·7 cm., height 0·8 cm., width 0·2 cm. The free margin of the right valve is poorly calcified and is similar to that of the smaller valve in *Aloidis* (Yonge, 1946). This margin of periostracum fits tightly against the marginal region of the left valve when the valves are closed. In addition, the margin of both valves is poorly calcified in the siphonal region.

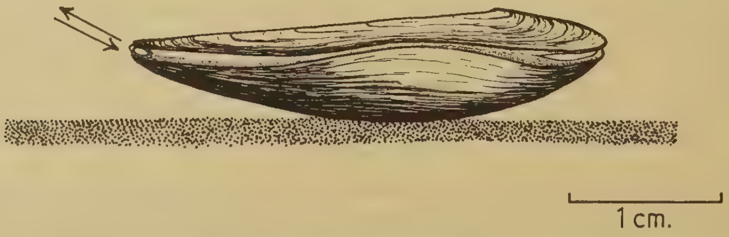
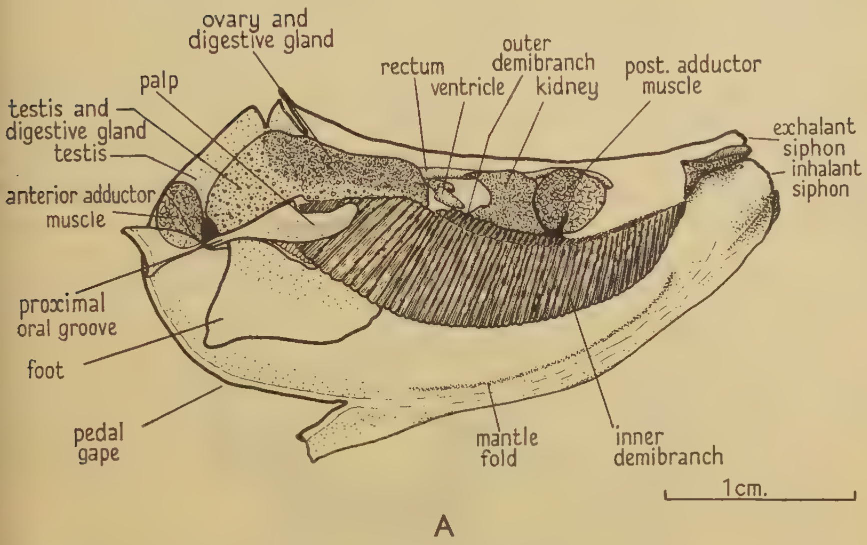


FIG. 1. *P. inaequivalvis* viewed from the free-margin and lying in its normal position on th left valve. The direction of the inhalent and exhalent currents are shown.

The hinge teeth are simple and consist of a single cardinal tooth in each valve. The tooth on the right valve is the more prominent and lies at right angles to the hinge margin. This fits into a well-defined socket on the opposite valve. The internal ligament lies posterior to the tooth and usually has a posterior extension. Unlike the Lyonsiidae, there is no lithodesma, but like the latter group the valves of *Pandora* are united dorsally by periostracum. This is probably a primitive feature (see Yonge, 1952; Owen, Trueman, and Yonge, 1953). The periostracum extends posteriorly almost to the siphons and anteriorly to the anterior adductor muscle.

There is extensive fusion of the mantle edges, the pedal gape occupying less than one-third of the ventral margin (fig. 2 A). Unlike the Lyonsiidae and the



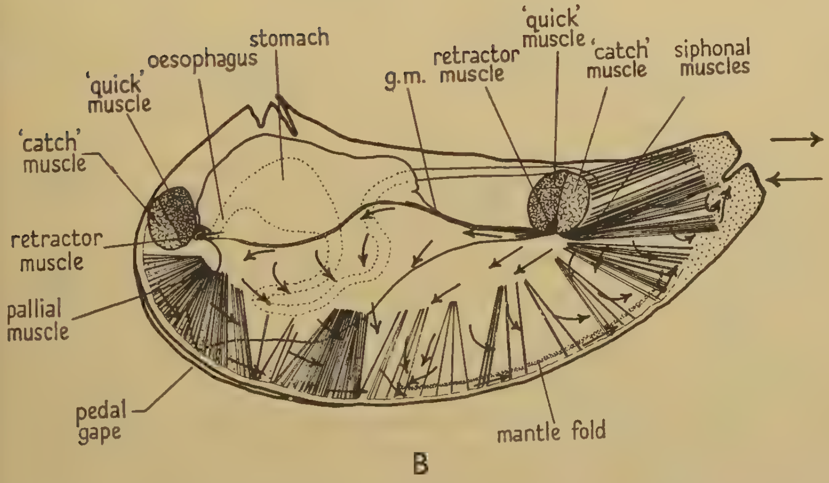


FIG. 2. A, lateral view of *Pandora* with the left valve removed and mantle cut away. B, lateral view of *Pandora* with the left valve removed to show the ciliary currents of the mantle and the mantle musculature. *g.m.*, line of attachment of the gill lamella.

Thraciidae there is no fourth pallial aperture. There is complete fusion of the tissues and not merely a cuticular junction (Atkins, 1937*b*). Both inner and middle lobes of the mantle edge are involved in tissue fusion.

The siphons of *Pandora* are short and are not capable of extending more than a few millimetres. There is no siphonal embayment such as is found in *Thracia*. The apertures are slightly lobed and are fringed with a number of short papillae. There is no well-defined periostracal groove and the periostracum is secreted here, as it is ventrally, by the epidermal cells in the region *F* shown in fig. 3. The periostracum is apparently secreted in a semi-fluid state

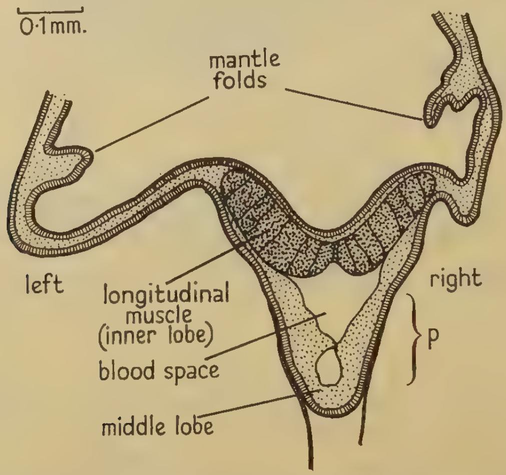


FIG. 3. Transverse section of the fused ventral mantle edge showing the position of the mantle folds. *P*, region of the secretion of the periostracum.

This is particularly the case at the base of the siphons where sand grains become incorporated in it. Yonge (1952) describes a similar condition in the Lyonsiidae where in the case of *Enteodsma* and *Lyonsia* the shell may be covered by sand grains attached to the periostracum. In *Pandora* the sand is restricted to the base of the siphons and is never found attached to the surface of the shell.

The pallial muscles are well marked. Anteriorly there are two well-developed groups in association with the pedal gape while posteriorly a similar group of muscles form the siphonal retractors. Between the pedal and siphonal groups there are scattered muscles controlling the fused mantle margins (fig. 2 B). The anterior and posterior adductor muscles are well developed and approximately equal in size. The 'quick' muscles are very well developed and, as in *Aloidis*, are probably associated with very large amounts of bottom material entering with the inhalent current (Yonge, 1946). The anterior and posterior pedal retractor muscles are unusual in that they are inserted by the *ventral* side of the adductor. The posterior retractor may be wedged between the 'quick' and 'catch' muscles.

A pair of mantle folds, dorsal to the fusion of the inner mantle lobes, extend rom a few millimetres behind the pedal gape to a position anterior to the in- alent aperture (figs. 2 and 3). The folds are not wide enough to form a roof as n *Spisula* (Yonge, 1948). The mantle currents are as shown in fig. 2, B. A main iliary tract for the rejected particles extends from a position opposite the tip f the palps posteriorly to the mantle fold. Particles are carried along the roove formed by the mantle fold and the fused inner lobes of the mantle. The cilia in the groove are longer ($70\,\mu$) than those of the general mantle sur- ace ($<40\,\mu$). The rejected particles do not collect at the ventral side of the nhalent aperture but are carried on to the lateral and dorsal walls. Particles alling on to the mantle from the heel of the foot and from the anterior portion of the gill lamella are carried to the anterior end of the mantle fold. Those rom the posterior end of the gill lamella are deflected ventrally and posteriorly and are not carried along the ventral groove. Dorsally, where the mantle and gill margins meet, the cilia beat anteriorly so that particles are carried anteriorly on to the gill lamella.

ORGANS IN THE MANTLE CAVITY

There is a moderately developed foot with a byssus groove and gland. The latter is not functional in the adult. The labial palps are of medium size and function in the usual manner. The dorsal edge of the palp is unusual in that it has numerous fine ridges running in the same direction as the normal palp ridges. The proximal oral grooves are long and deep. They run forward on either side of the foot and, anteriorly, the outer sides of each groove unite to form a hood over the mouth.

The ctenidia are plicate and heterorhabdic; *P. pinna* has nine filaments to each plica and *P. inaequivalvis* seven, confirming the observations of Ridewood (1903). The outer demibranch is very much reduced. It is reflected dorsally and only the direct lamella remains. Atkins (1937*a*) has suggested that as there is a relatively small number of filaments to each plica it might be expected that the sorting mechanism will be different from that of the *Pinna* type which she describes and figures (pp. 347 et seq.). But examination shows that the sorting mechanism is the same as in the deeply plicate species.

All frontal cilia beat towards the free margins of the inner demibranch, those on the outer demibranchs beating towards the axis and so to the outer surface of the inner demibranch. The marginal groove is scalloped and fine particles moving down the principal filaments are carried into the depth of the marginal groove. Larger particles, that cannot reach the principal filaments because of the narrowness of the interplical space, arrive at the gill margin and are carried anteriorly outside the groove along the tips of the filaments (Atkins 1937*a*, p. 347). Many of these will then fall on to the foot and mantle. The gills are sufficiently muscular for the opposing tips of the apical filaments to close over the marginal groove. Examination under high power of the edges of the apical and adjacent ordinary filaments shows that sorting of the particles also occurs here. The long terminal cilia which move the larger particles anteriorly extend only over two-thirds of the tip of the filament while the remainder are unmodified frontal cilia. The latter allow any fine particles to pass into the marginal groove (fig. 4.). A similar sorting mechanism has been described by Yonge (1946) in the case of *Aloidis*.

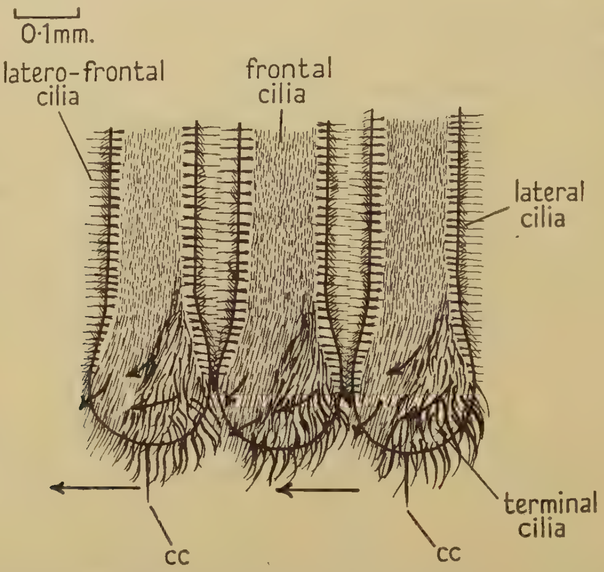
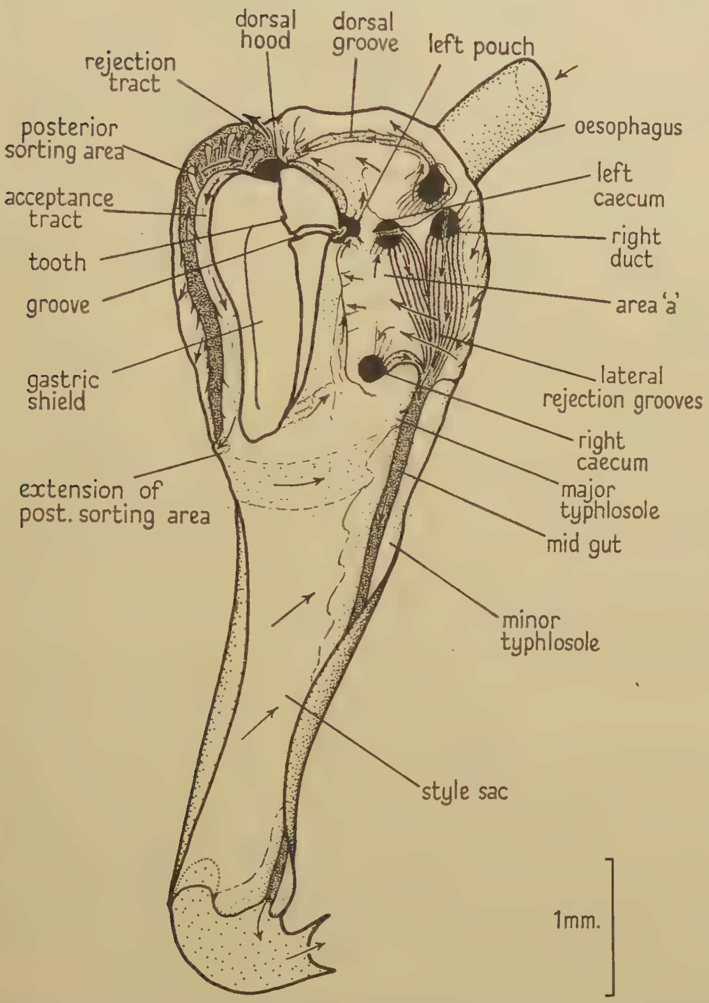


FIG. 4. Sorting mechanisms at the edge of the apical and ordinary filaments of the inner gill lamella. *CC*, coarse cirrus-like cilia.

ALIMENTARY CANAL

The course of the alimentary canal is indicated in figure 2, B. A short oesophagus leads into a relatively large stomach. The mid-gut and style sac are in communication, the intestine curves anteriorly, ventral to the stomach and then loops posteriorly and dorsally to terminate posterior to the posterior adductor. The ventricle lies ventral to the hind gut. The stomach was dissected from various aspects and with the aid of carmine and fine carborundum particles the ciliary currents investigated. The nomenclature proposed by Graham (1949) and Owen (1953) has been followed.

The morphology of the stomach and the ciliary currents can be followed with reference to fig. 5. Particles accepted by the palps are carried through the oesophagus within a string of mucus which is wrapped around the tip of the crystalline style. The style is relatively large in *Pandora*—0·5 cm. in a specimen 3·2 cm. in length—and it rotates in a clockwise direction when viewed from the oesophageal end of the stomach. It is prevented from entering the dorsal hood by means of the tooth that is present on the recurved portion of the gastric shield. The gastric shield is held in place by flanges extending into the dorsal hood and left pouch.



IG. 5. The stomach opened from the right side to show the direction of the ciliary currents.

The ciliary mechanisms of the dorsal hood and the posterior sorting are
are similar to those described by Owen (1953) for *Glossus*. Released particle
from the tip of the crystalline style are carried, probably by the revolvin
style, on to the posterior sorting area which is moderately well develope
with a small backward extension ventral to the posterior end of the acceptanc
tract. The cilia of the grooves and ridges of this area exercise a quantitativ
selection; lighter particles are carried into the dorsal hood while heavie
particles are conveyed to the rejection tract. The rejected particles are conveyed to the mid-gut by way of the lateral rejection grooves (fig. 5).

The dorsal hood, which is of moderate size, is directed anteriorly and to th
left. Accepted particles are carried to the tip of the hood, those on the dors
side being passed on to the acceptance tract while those on the ventral sid
are carried on to the flange of the gastric shield and the head of the style (se
Owen, 1953, fig. 8.). Fine particles are conveyed posteriorly by the acceptanc
tract which lies along the dorsal side of the gastric shield. These particles ar
then carried ventrally over the posterior wall of the stomach to the right an
left caeca and to the left pouch. This movement across the posterior wall is i
an opposite direction to that of the style, i.e. in an anticlockwise directio
when viewed from the anterior end of the stomach. Particles are carrie
either directly to the openings of the digestive ducts or fall on to the are
A next to the ventral and lateral side of the gastric shield. In the latter cas
those particles near the right caecum are carried towards its aperture, whil
the remainder are directed towards the gastric shield to a poorly define
groove and then anteriorly to the left pouch.

The stomach of *Pandora* is unusual in that the major typhlosole is ver
poorly developed. In many of the specimens examined it terminated jus
within the entrance to the right caecum and in the remainder, just before th
entrance. It does not continue towards the left caecum and form a flap-lik
cover to an intestinal groove as it does in *Glossus* (Owen, 1953) and othe
Eulamellibranchia (Graham, 1949). In place of an intestinal groove there
a series of ten to fifteen grooves (lateral rejection grooves) which extend fro
the openings of the right duct and the right and left caeca to the mid-gu
Rejected particles from these ducts are carried along the grooves. In addition
cilia on the crests of the ridges between these grooves beat ventrally, conveyin
accepted particles towards the area *A*.

The path of rejected particles from the left pouch is not clear. Carefu
examination shows no rejection path joining those from other ducts. The onl
material observed leaving the pouch was carried on to the flange of the gastri
shield. Apparently rejected material returns to the general circulation of th
stomach and is probably pulled over the groove lying below the tooth of th
gastric shield and so on to the style.

Particles failing to enter the digestive ducts are carried towards the oesophageal region of the stomach. These particles, together with any stra
particles entering by the oesophagus, are carried dorsally on either side of th
oesophageal opening to the dorsal groove. From here they are carried to th

se of the dorsal hood. Some of these particles are carried directly to the ght duct.

The differences between the stomach of *Pandora* and those of other ulamellibranchia are a possible consequence of elongation. The right and ft caeca of *Pandora* are widely separated and, as the poorly developed major phlosole extends no further than the right caecum, there is no flap covering intestinal groove. Instead a series of lateral rejection grooves are present hich function in a similar way to the ridges and grooves of the posterior rting area. Thus, while rejected material is carried in the grooves, accepted rticles are passed across this region by the cilia on the crest of the ridge etween each adjacent groove.

It seems probable that the area *A* is homologous to that portion of the omach in other Eulamellibranchia, e.g. *G. humanus* (see Owen, 1953, p. 98), at lies between the gastric shield and the flap of the major typhlosole.

DISCUSSION

Pandora is apparently adapted for life on the surface of the substratum. . *inaequivalvis* was found to be abundant on *very* sheltered and slightly elving beaches around L.W.S.T. level. No buried specimens were found, ut algal growth confined to the siphonal region suggests that *Pandora* is metimes buried with only the siphons showing. The animals were not bserved to burrow either in the field or in the laboratory, although there is a oderately developed foot and the pedal gape is sufficiently wide for this foot be extended. Although no observations have been recorded, it is possible at the foot is used to uncover and orientate the animal if it becomes silted ver.

The shell is markedly inequivalve and the animals lie on the surface on the ft, convex side. The siphons are very short and never extend more than a w millimetres. They are directed upwards at an angle to the substratum nd not horizontally along the surface (fig. 1). Even so, large quantities of diment will almost certainly be taken into the mantle cavity. This is re- oved by the sudden contractions of the well developed 'quick' portion of the dductor muscles.

In addition lateral compression in *Pandora* is very great. This may be why e retractor muscles lie in the unusual position ventral to the adductor uscles.

This, also, may be why the ctenidia only extend over the posterior portion f the visceral mass, and thus there are necessarily long proximal oral grooves resent.

I wish to express my thanks to Professor C. M. Yonge, F.R.S., for his en- ouragement and help, and to my wife for her help and criticism. I also wish thank Mr. G. Owen for his valuable suggestions and criticism of the section ealing with the stomach. I am indebted to Professor P. Drach and the Staff of the Station Biologique, Roscoff, for their kind assistance. This work wa made possible by a grant from the John Murray Committee of the Roy Society.

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The Morphology of Peripheral Nerve Terminations in the Skin

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With three plates (figs. 1, 2, and 5)

SUMMARY

1. An account is given of the innervation of the skin from selected regions of the body in man, the rhesus monkey, and the rabbit. It is based on a critical evaluation of methylene blue and silver preparations in skin which had been treated with the enzyme hyaluronidase before removal from the body and in skin which had not thus been treated.

2. The nerve fibres and terminals in preparations treated with hyaluronidase resemble those seen in fresh specimens of cornea under phase contrast conditions.

3. The numerous artifacts seen in the cornea following methylene blue staining or silver impregnation, the origin of which is known, resemble those seen in specimens of skin not treated with hyaluronidase.

4. It is shown that cutaneous nerves ultimately terminate in an arborization of fine naked axoplasmic filaments. The naked filaments always arise from ensheathed stem fibres of varying diameters and they all end freely and extracellularly in relation to a variety of tissue elements in the skin.

5. The actual terminal filaments wherever they end in relation to the epidermis, dermal connective tissue, blood-vessels, sweat glands, hair follicles, or within capsules of epithelial cells, cannot be distinguished from one another on the basis of inherent morphological differences.

6. Terminal filaments from neighbouring stem fibres are always intimately related to one another and to the tissues in which they lie.

7. The stem fibres and axoplasmic filaments to which they give rise in encapsulated endings and hair follicles are so arranged that the slightest deformation of capsule or of follicle will alter the relationship of the terminal filaments to their stem fibres.

8. Encapsulated nerve-endings may be served by more than one stem fibre but none of the terminals of these fuse with one another. Likewise, the terminals derived from individual stem fibres ending in relation to hair follicles do not enter into protoplasmic continuity with one another.

9. The number and length of the axoplasmic filaments varies greatly from stem fibre to stem fibre.

10. Our observations on the behaviour of the cutaneous nerve fibres and their terminals under different histological conditions provides us with a standard by which we can distinguish artifacts and thus re-evaluate the observations on cutaneous innervation which have been reported in the literature.

11. Evidence is brought forward which suggests that the naked axoplasmic filaments derived from sensory stem fibres may be responsible for 'flare' and other 'nocifensor reactions' (Lewis), in addition to and apart from their sensory functions.

12. The relationship of these observations to the mechanism of cutaneous sensibility is discussed.

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INTRODUCTION

IN 1950 Weddell and Zander, using the cornea, compared and contrast the appearances observed following a number of different neurohistologic techniques with those seen in fresh specimens under phase contrast condition This enabled them to distinguish between artifacts resulting from the tec nique, and the appearance of the nerve fibres and their terminations in the u damaged state. The morphological details of neural elements, either in tissu in which they are not so readily distinguishable under the phase contra microscope, or in tissues which are obtained from living human subjects st had to be inferred by analogy with the behaviour of the corneal nerves whe submitted to similar selective histological methods. Weddell and Pallie (1954 however, have found that hyaluronidase, or 'spreading factor', can be used i such a way that the tissue neural elements can be displayed by the common employed neurohistological techniques in an almost undistorted form an relatively free from artifacts.

The possibility of re-examining the mode of termination of cutaneous nerv in the light of these developments at once presented itself. A survey of t literature revealed that, despite the existence of a vast number of publication there is no agreement whatever as to the nature and arrangement of the neur elements in skin (Weddell, Palmer, and Pallie, 1954). In this paper, therefor we shall present an account of the innervation of skin and discuss the bearin of our observations on the present conception of the mechanism of cutaneou sensibility.

MATERIAL

1. *Human skin.* Not less than three specimens from different subjects wer obtained at operation from the following regions: pinna, chest wall, abdomen front of leg, back of arm, front of forearm, lip, and anal margin. Skin wa obtained from the finger pads, glans penis, glans clitoris, and labium majus i three separate subjects within four hours of death.

2. *Macaque monkey.* Skin from similar regions to those from which it ha been obtained in human subjects was removed from two anaesthetized animal

3. *Rabbit skin.* Skin was obtained at operation from the dorsum of the ea in forty-five animals and from the upper lip in ten animals.

In every case the skin was removed with care and every effort was made t reduce the amount of handling and, in particular, stretching and other form of mechanical distortion to a minimum.

METHODS

Alternate specimens of skin were infiltrated with hyaluronidase (Wedde and Pallie, 1954) and either stained by local injection of 0·02–0·04 per cent methylene blue or removed and fixed in formalin preparatory to impregnatio with silver by a modification of the Bielschowsky–Gros method.

OBSERVATIONS

Our observations fall into two series. In the first part we shall describe the innervation of skin as seen after treatment with the enzyme hyaluronidase and in which the staining or impregnation was optimal. In the second part we shall compare and contrast these observations with those seen in skin which had not been treated with hyaluronidase.

I. TREATED SKIN

In every specimen treated with hyaluronidase (from whatever species and from whatever region of the body it had been obtained), all the cutaneous nerves ultimately terminated in fine freely ending axoplasmic filaments, less than 1 μ in diameter, which are naked, that is, having no continuous cellular or connective tissue sheath around them. The pictures seen after methylene blue staining and silver impregnation by either method are similar in this respect.

It was observed that the patterned arrangement of the filaments varies from species to species and from region to region in the same species. These variations, although striking, can be analysed in terms of units which are morphologically identical.

It is chiefly from the point of view of the appearance of the terminal units that we shall describe the innervation of the skin. We shall comment on the patterned arrangement of the units, but we shall not attempt to compare skin from different regions or from different species in this respect.

Nerve fibres only terminate in the skin after passing through the cutaneous nerve plexus in which they usually branch and always ramify extensively. The preterminal or stem fibres which arise from the plexus are themselves almost always branches of parent fibres. The diameter of the stem fibres varies considerably and more than half of them are myelinated. On the other hand, some myelinated stem fibres lose their myelin sheath and travel for a distance of several millimetres as non-myelinated fibres before terminating. It is characteristic that all preterminal or stem fibres possess a sheath of Schwann elements.

After travelling for distances which vary from region to region and from species to species (it is commonly 0·5 mm. in the rabbit), the stem fibres give rise to a profuse arborization of naked axoplasmic filaments. The filaments often appear to end in small expansions, although this is by no means always the case. In addition, the filaments are often beaded rather than smooth in outline. However, the appearance of a series of lighter and darker dots simulating beading may be an optical effect due to alterations in the plane in which the filaments lie as they pass in between cells and other tissue elements.

For the purposes of description it is convenient to consider the terminals arising from stem fibres in the skin as being either spread over a wide area or concentrated within a small zone. Further, so that our observations can be conveniently related to those in the literature, we shall describe them under three headings: (1) unencapsulated nerve-endings; (2) nerve-endings related to hair follicles; (3) encapsulated nerve-endings. Nevertheless, we want to emphasize that this kind of classification is purely arbitrary; the terminals springing from neighbouring stem fibres are so intimately related to one another and to the tissue elements among which they lie that any simple categorization on a morphological basis such as this may be very misleading.

I. *Unencapsulated nerve-endings*

Stem fibres of all sizes giving rise to naked axoplasmic filaments can be seen throughout the thickness of the skin. The actual filaments have no distinguishable morphological characteristics but their number, length, and disposition relative to one another varies from place to place in skin from similar regions. They probably also vary in this respect in skin from different regions of the body but they are too numerous, too fine, and arranged in such a complex manner that we cannot give any useful estimate of this. The patterned arrangement of the terminals, however, clearly conforms to the patterned arrangement of the tissue elements among which they lie. For instance, stem fibres which are commonly myelinated can be seen to give rise to a profuse arborization of fine naked axoplasmic filaments radiating in all directions in a plane immediately subjacent to the basal layer of the epidermis. They give rise at frequent intervals to filaments which enter the epidermis and ramify among the cells of the *stratum granulosum* where they terminate freely, often in small swellings. No quantitative estimates of the area of skin subserved by a single stem fibre can be given, for the areas involved are variable and the terminals from neighbouring stem fibres intertwine so closely with one another that the technical difficulty of direct measurement is almost unsurmountable. However, the minimal area which has been measured was around 1 sq. mm. and fibres have been traced for distances which suggest that areas greater than 1 sq. cm. may be covered by filaments derived from a single stem fibre. We have never seen axoplasmic filaments within the cytoplasm of a cell-body and the axoplasmic

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FIG. 1 (plate). A, axoplasmic filaments ending among the cells of the epidermis in a rabbit ear. Some of the irregularities are of an optical nature and due to abrupt changes in direction of the filaments. Methylene blue preparation.

B, stem axon giving rise to axoplasmic filaments ending among epidermal cells in rhesus monkey skin. The arrow is pointing to a terminal derived from an axoplasmic filament situated in another optical plane. Silver preparation.

C, axoplasmic filaments ending in the superficial layers of the dermis of human abdominal skin. Methylene blue preparation.

D, axoplasmic filaments ending in relation to a small cutaneous arteriole in rabbit ear skin. Methylene blue preparation.

E, axoplasmic filaments ending in relation to the coils of a sweat gland in the pad of a rhesus monkey finger. The gland cells could only be identified under phase-contrast conditions. Methylene blue preparation.

F, axoplasmic filaments springing from stem fibres ending in relation to a hair follicle in rabbit ear skin. The filaments are so fine that at this magnification they are difficult to visualize as separate entities. Methylene blue.

G, axoplasmic filaments springing from stem fibres ending in relation to a hair follicle in rabbit ear skin. The axoplasmic filaments are swollen and somewhat irregular in outline in comparison with those in methylene blue preparations. Silver preparation.

H, circular and vertical formations of axoplasmic filaments ending in relation to a hair follicle in rabbit ear skin. Methylene blue preparation.

I, vertical and circular formations of axoplasmic filaments ending in relation to a hair follicle in rabbit ear skin. Methylene blue preparation.

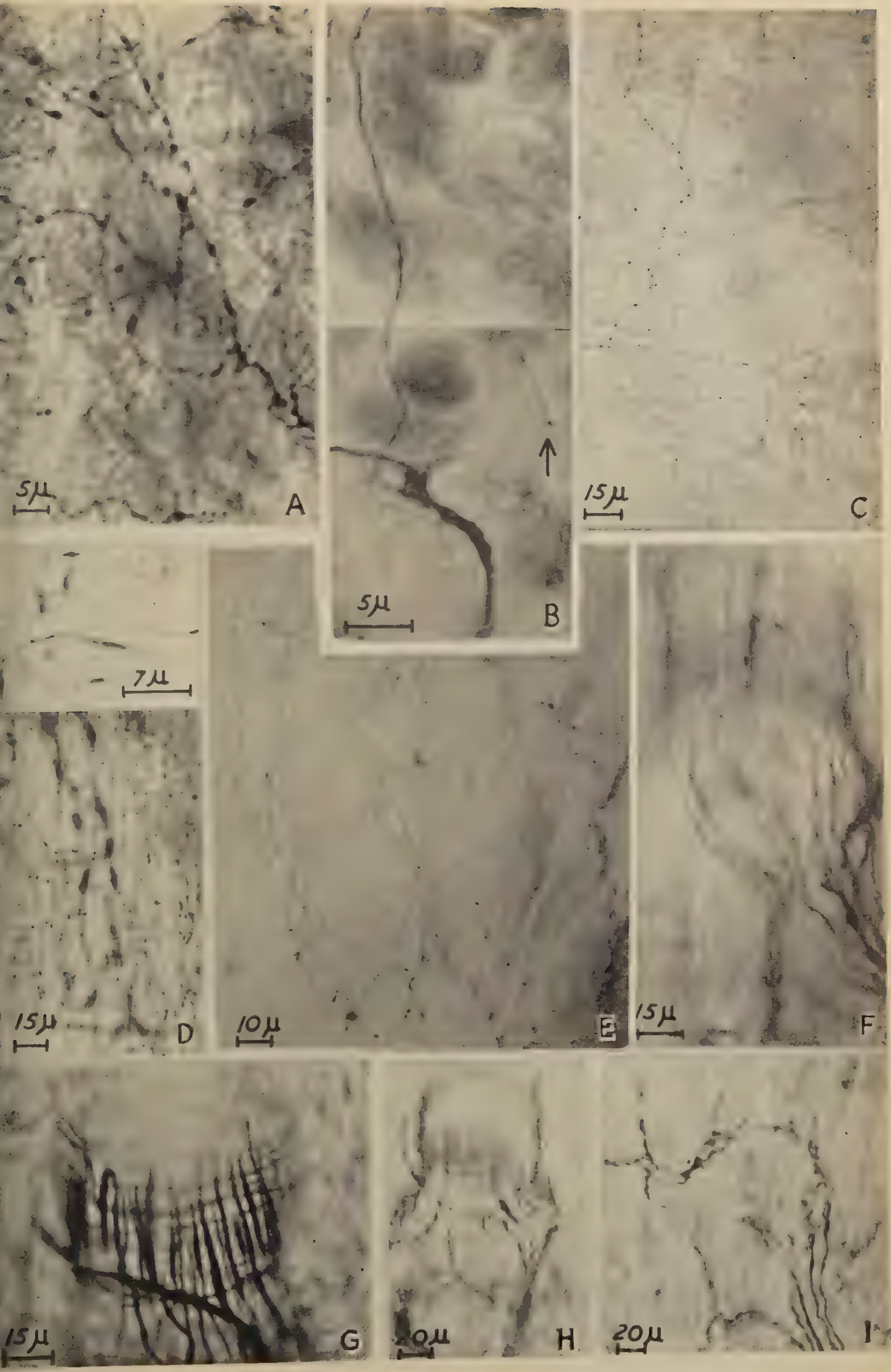

FIG. 1

G. WEDDELL, W. PALLIE, *and* E. PALMER

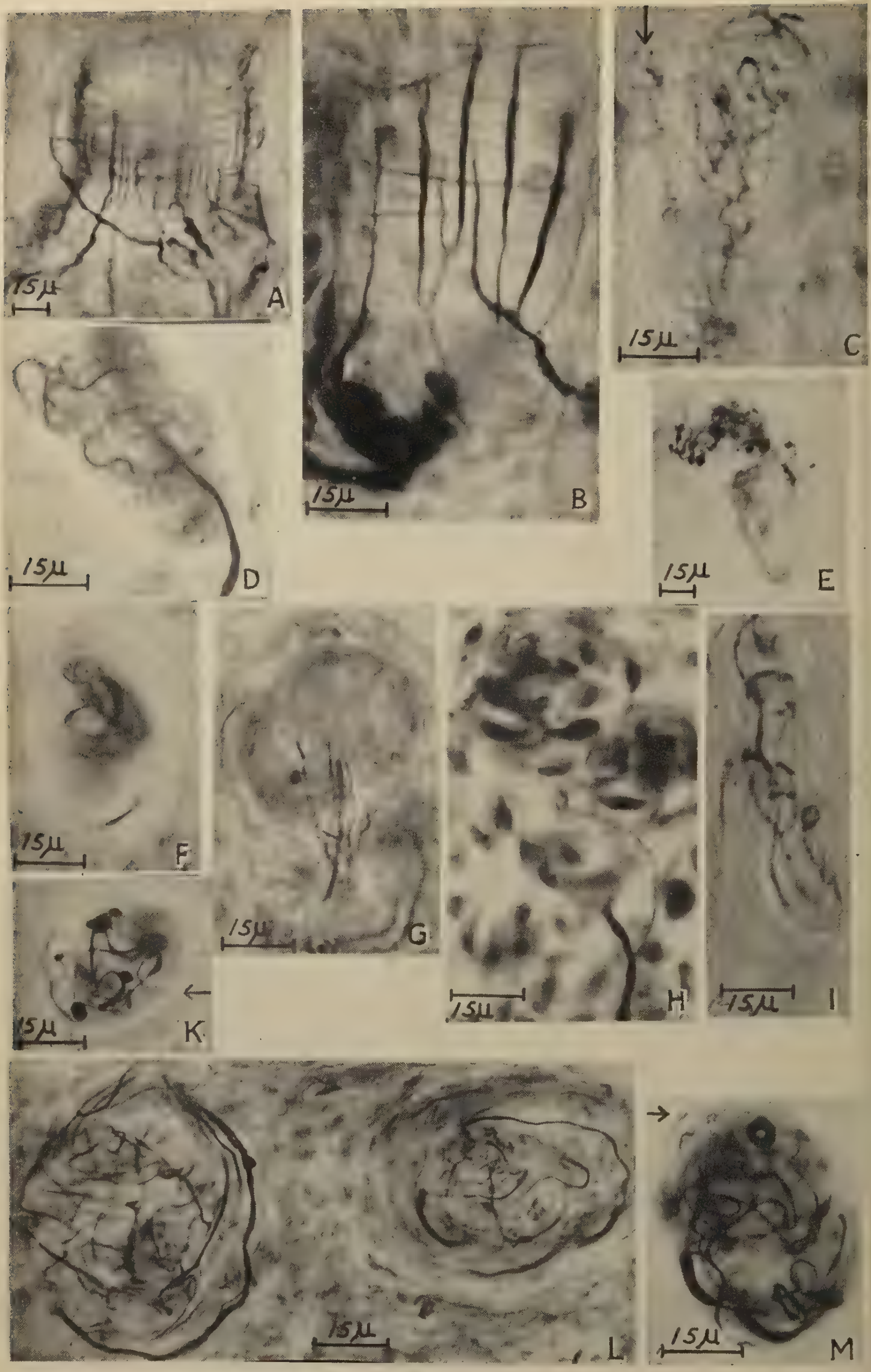

FIG. 2

G. WEDDELL, W. PALLIE, *and* E. PALMER

laments derived from the same or neighbouring stem fibres are never observed to fuse with one another to form protoplasmically continuous nerve nets ig. 1, A and B).

Unencapsulated nerve-endings, or stem fibres ending in arborizations of aked axoplasmic filaments, are also found in the dermis. Neither the stem bres nor the filaments to which they give rise lie in a single stratum but are cattered throughout the dermis. The filaments neither lie nor terminate in any onstant relationship to any particular tissue element, but the filaments are most numerous where the arterioles and capillaries are most abundant (Fig. , C). Filaments derived from neighbouring stem fibres travel in so many different directions and intertwine so closely with one another that it has not proved possible to determine the relative extent of dermis subserved by the ilaments derived from a single stem fibre. It is only in the thin skin from the dorsum of the rabbit ear that we can be certain that all the terminals end freely, or in thicker skin, such as that from the finger pad of the rhesus monkey,

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FIG. 2 (plate). A, hair follicle from rabbit ear skin showing mode of formation of axoplasmic ilaments from stem fibres. Methylene blue preparation (cf. fig. 3).

B, hair follicle from rabbit ear skin showing mode of formation of axoplasmic filaments from tem fibres. Some of the pre-terminal axons are somewhat swollen and irregular. Silver preparation (cf. fig. 3).

C, 'encapsulated nerve-ending' from the labium majus of a rhesus monkey. Stem fibres on he right give rise to a complex mass of fine axoplasmic filaments which terminate freely within he capsule, the cells comprising which could only be seen under phase-contrast conditions. Note the arrow pointing to unencapsulated terminals ending in relation to the sub-epidermal tissues. Methylene blue preparation.

D, 'encapsulated nerve-ending' from human glans clitoris. The stem fibre is giving rise to a complex mass of fine axoplasmic filaments which terminate freely. Silver preparation.

E, 'encapsulated nerve-ending' from monkey finger pad. The stem fibre gives rise to a complex mass of fine axoplasmic filaments, some of which have terminal swellings. Methylene blue preparation.

F, 'encapsulated nerve-ending' from human finger pad; specimen obtained 4 hours after death. The stem fibre gives rise to a complex mass of fine axoplasmic filaments, some of which can be seen to be surmounted by terminal swellings. Silver preparation.

G, 'encapsulated nerve-ending' from human glans clitoris. Stem fibres are giving rise to a complex mass of extremely fine axoplasmic filaments. This figure should be compared with ig. 4. Silver preparation.

H, 'encapsulated nerve-ending' from human glans clitoris; specimen obtained 4 hours after death. A stem fibre is giving rise to numerous fine axoplasmic filaments. Their relation to the cellular elements of the capsule can be seen in this preparation. Silver preparation.

I, 'encapsulated nerve-ending' from monkey glans clitoris. The discrete nature of the terminal axoplasmic arborizations can be appreciated in this preparation. Methylene blue preparation.

K, 'encapsulated nerve-ending' from human finger pad; specimen obtained 4 hours after death. Some of the axoplasmic filaments have disintegrated and silver has been deposited in blobs. The arrow points to an undistorted terminal filament. Silver preparation.

L, 'encapsulated nerve-ending' from a human glans clitoris. Stem fibres can be seen giving rise to a profusion of fine discrete axoplasmic filaments. Silver preparation (cf. fig. 4.)

M, 'encapsulated nerve-ending' from a human finger pad; specimen obtained 4 hours after death. The stem fibres can be seen giving rise to a number of fine discrete axoplasmic filaments ending in relation to the surface of the capsular cells (which can only be seen under phase-contrast conditions). The terminals are somewhat distorted for they appear to end in rings. This picture should be compared with those given of 'boutons terminaux', ending in relation to cells within the central nervous system. Silver preparation.

some of the terminals cannot be traced to their final destination even in who cleared methylene blue-stained specimens. Since, however, nerve termina are never seen to fuse with one another in the dermis of rabbit ear skin, ther seems no reason to believe that fusion occurs in skin from other species an from other regions of the same species. In general, as in the case of th epidermis, the stem fibres giving rise to the naked terminals in the derm approach the skin surface from below and the axoplasmic filaments to whic they give rise arborize into different connective tissue strata which lie parall to the stratum germinativum.

Other stem fibres, most commonly of the non-myelinated variety, leave th cutaneous nerve plexus in bundles of four to six fibres and proceed towards th arterioles.Having reached the adventitia they run parallel to the vessel for lon distances, being joined by further bundles of stem fibres from time to time. A intervals along the course of the vessels, the stem fibres give rise to, or end i an arborization of fine naked axoplasmic filaments which terminate freely often in slight enlargements, on the surface of the smooth muscle fibres a right angles to their long axis; they do not end in relation to any specific pa of the muscle cells. Axoplasmic filaments from neighbouring stem axons overlap and intertwine so closely with one another and, moreover, such a larg number can be brought into focus in a single plane under low magnificatio that they give the appearance of a continuous nerve net (Fig. I, D, lower half Under higher magnification, however, it can be seen that the filaments a terminate freely and no protoplasmically continuous nerve nets can be detecte (fig. I, D, upper half).

Venules are similarly innervated but fewer stem fibres are involved; th density of the intertwining filaments is also less in proportion to the decreas in density of smooth muscle fibres.

Still other stem fibres, both myelinated and non-myelinated, leave th cutaneous plexus, and proceed towards the sweat glands. On reaching th capsule of the gland they give rise to an arborization of fine axoplasmi filaments which intertwine with their neighbours to form a basket-lik envelopment of the gland coils. Many filaments can be seen to terminate i the adventitia; other filaments terminate on the surface of and in between th gland cells, often in small terminal swellings (fig. I, E). In methylene blu preparations the relationship of the terminals to the various layers composin the sweat gland coil is most clearly seen under phase-contrast conditions.

2. *Nerves ending in relation to hairs*

Stem fibres leave the cutaneous plexus and approach the hair follicles i bundles which vary in number from two to five, depending upon the size o the hair, and they approach the hair from different directions. In thin skin such as that from the rabbit ear, they look rather like the spokes of a whee embedding themselves in the hub. In thicker skin, the fibres take a mor oblique course towards the follicle. In the case of large hair follicles in th rabbit ear, as many as twenty-eight stem fibres (some arising from a commo parent fibre) have been counted in the nerve bundles which approach the follicle. The stem fibres are myelinated and, in the case of the large hairs (fig. 1, F) they are amongst the largest seen in the skin. They reach the hair follicles just below the point at which the sebaceous glands open into them. They then give rise to numerous branches which run in the outer layer of the dermal coat parallel to the hair shaft. One series of stem fibres change direction relatively abruptly and pass towards the middle layer of the dermal coat in which they lose their sheaths and give rise to a series of fine naked axoplasmic filaments which encircle the hair where they terminate freely amongst the collagen bundles (fig. 1, G and H). The remaining stem fibres continue towards the skin surface but, at the same time, approach the vitreous layer in a plane deep to the layer of circular fibres just described. These stem fibres lose their sheath as they pierce the vitreous layer of the follicle to give rise to a series of fine naked axoplasmic filaments which terminate freely among the cells of the outer root sheath (fig. 1, G and H; fig. 2, A and B). At no time have we seen any connexions between these two sets of nerve fibres and terminals which lie so closely one within the other. Fig. 3 shows in diagrammatic form the terminal arrangements in a hair follicle and demonstrates clearly that mechanical deformation of the hair by bending, pressing, or pulling will affect the relationship of the axoplasmic filaments to the stem fibres from which they arise.

The size of the hairs and the hair follicles varies in skin from different regions of the body and from place to place in the same region. In general, the number of stem fibres and wealth of axoplasmic filaments to which they give rise is directly related to the size of the follicle but not necessarily to the size of the hair. During periods of active hair growth, however, parts of the follicle appear disproportionately large.

We have not seen any nerve fibres or terminals specifically related to the tissues of the hair bulb. As we have already said, there are nerve fibres and terminals disposed throughout the dermis and some of these come into close association with the hair bulb, but no specific relationship can be demonstrated.

3. *Encapsulated nerve-endings*

Encapsulated nerve-endings are seen in large numbers in mucous membranes and in skin devoid of hairs. They are not seen in skin covered with hairs but they are seen in skin in which the hairs are sparse, such as that covering the lower part of the forearm. Encapsulated nerve-endings and hair follicles resemble one another in one important respect; they are both associated with stem fibres of large diameter which give rise to aggregations of naked axoplasmic filaments which are sharply circumscribed. Encapsulated nerve-endings vary in size, in shape, and in their situation relative to the skin surface from area to area in the same region, and from region to region of the body (fig. 2, C–G and I). Unlike hair follicles, however, the number of stem fibres associated with each capsule is relatively much fewer; the largest number we have seen is three and more often only one stem fibre is involved (fig. 2, G).

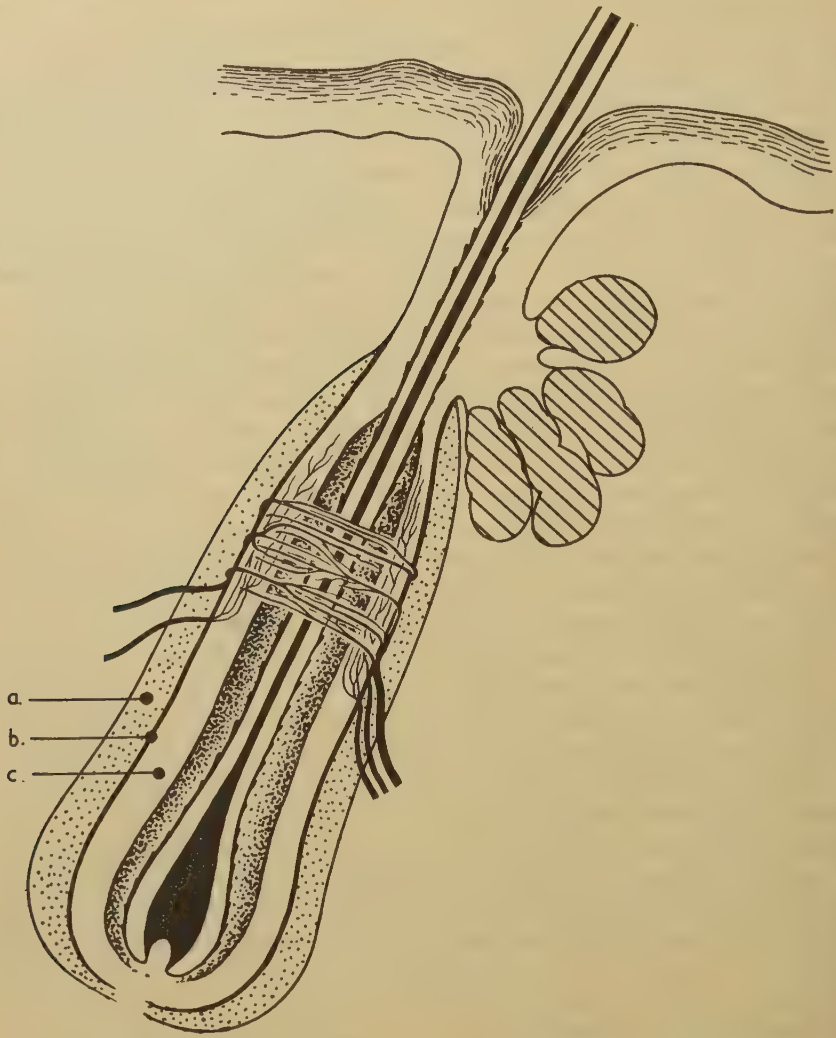

Fig. 3. Shows, in diagrammatic form, the manner in which nerves terminate in relation to hair follicles. The relationship of the stem fibres to their terminals will be altered if the hair is bent, pulled, or pushed. (*a*) dermal coat, (*b*) vitreous membrane, (*c*) outer root sheath [For the sake of clarity, outlines of cell boundaries have been omitted.]

The course pursued by the stem fibre or fibres within the capsule may be simple or extremely tortuous. In addition, the capsule itself may be a comparatively simple collection of cells of varying size forming a homogeneous mass among which the delicate axoplasmic filaments derived from the stem fibres are distributed; or it may be a more complicated structure in which there are clusters of cells of varying size grouped together in an irregular manner; or, again, it may be any intermediate form between these two extremes (fig. 2, D, G, H, and L).

We found it impossible to classify these endings into morphologically distinct groups on the basis of either the arrangement of the capsular cells or of the stem fibres within the capsule, for there are so many intermediate forms. Nevertheless, that there are regional differences in the morphology of encapsulated endings cannot be denied. For instance, endings in the finger pulp tend to be large and complicated and to conform in shape to the skin papillae in which they are situated, whereas in the glans penis, glans clitoris, and anal mucous membrane the endings are less regular in size and shape and the stem fibres pursue a much more varied course within the capsule (fig. 2, E–H).

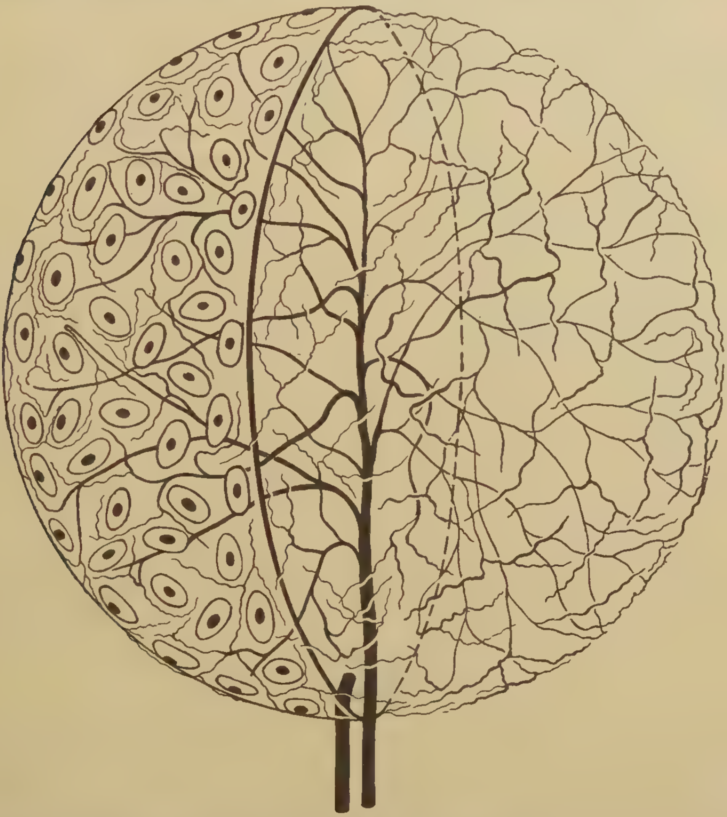

FIG. 4. Shows, in diagrammatic form, the manner in which nerves terminate in relation to encapsulated nerve-endings. The terminals are arranged approximately parallel to the surface of the capsule and at right angles to their stem fibres. Their relationships will be disturbed by the slightest deformation. [For the sake of clarity, outlines of cell boundaries have been omitted.]

Attempts to classify end-organs along these lines led us to the view that we were in danger of classifying regional differences in the morphology of the skin or mucous membrane as much as that of the encapsulated nerve-endings.

Despite the gross differences in size, shape, and complexity of axonal disposition, the actual terminations within the capsules all take the same form.

The stem fibres pursue a more or less tortuous course within the capsule and often branch repeatedly (fig. 2, D, K, L, and M). Leaving the stem fibre or fibres at intervals along their course and at their terminations within the capsule are a profusion of relatively short fine naked axoplasmic filaments which terminate freely among the cells of the capsular wall (fig. 2, C and E; H–M). The filaments never enter the cytoplasm of the cells nor do they fuse with neighbouring filaments; they sometimes end in small terminal swellings (fig. 2, C, E, H, I, and M). In certain cases, stem fibres pierce the capsular walls and give rise to a series of fine naked axoplasmic filaments which terminate freely either in the dermis or in relation to the epidermis, some of the filaments entering the stratum granulosum. In other cases, axoplasmic filaments pierce the capsular wall and terminate freely in the dermis.

In methylene blue preparations, owing to the highly specific nature of the stain it is sometimes difficult to distinguish encapsulated nerve-endings from unencapsulated free terminals, for the capsular cells cannot be seen (fig. 2, C). The difficulty can sometimes be resolved by switching to phase-contrast conditions when the capsular cells are rendered visible together with the axoplasmic filaments. In silver-impregnated specimens this difficulty does not arise so often, for the capsular cells can usually be seen though they are only faintly impregnated. Fig. 4 shows in diagrammatic form the manner in which nerves terminate in relation to an encapsulated ending. It demonstrates that the filaments are so arranged that they lie in layers in between the cells approximately parallel to the surface of the capsule and at an angle to their stem fibres. Their arrangement is such that deformation must lead to an alteration of the relationship between the stem fibre and the terminals to which they give rise.

II. UNTREATED SKIN

It is not possible to give a general description resulting from the use of both methylene blue staining and silver impregnation, for the pictures obtained are in some respects in sharp contrast. We shall therefore start by giving a description of the appearances following methylene blue staining. We shall follow the same order as that in our previous series of observations so that they can be compared and contrasted.

A. *Methylene blue staining*

1. *Unencapsulated nerve-endings*

(*a*) *Nerves ending in relation to the epidermis.* It is more difficult to stain naked axoplasmic filaments in skin which has not been treated with hyaluronidase. Intra-epidermal endings are only seen if the dye is injected intradermally and are best seen if the conditions demand that a certain amount of pressure has to be exerted to inject the dye. However, experience and strict attention to detail has enabled us to produce more specimens in parts of which the nerve fibres and their terminals appear as they do following treatment with yaluronidase. The dye has to be presented to the skin rapidly and in sufficient concentration to stain the axons without killing them while at the same ime care must be taken to ensure that it is not removed too rapidly by the lood-stream. In addition, damage to the skin must be avoided and during the ourse of processing for microscopic examination shrinkage must be reduced o a minimum. However, despite every precaution we are never able to achieve niform or consistent results throughout any particular preparation.

In every specimen a proportion of the nerve fibres and terminals exhibit ome degree of distortion and even a single axon, if traced from the cutaneous lexus to its termination, may behave differently in one plane as compared with another (fig. 5, A).

In a number of specimens stem fibres appear to give rise to fine terminals which, although usually more coarsely beaded, end in exactly the same way as they do in skin treated with hyaluronidase, but such appearances are not common and are never seen uniformly over a wide area of skin. In neighbouring regions in the same specimen of skin, the fine beaded fibres may appear to fuse with one another in the form of terminal nets lying within or just beneath the epidermis (fig. 5, B). In still other regions, the stem fibres may appear to end abruptly in terminal swellings situated just beneath the epidermis. These swellings are either solid and crescent-shaped or in the form of circumscribed reticulated nets of fine neural strands (fig. 5, C).

In some parts of the skin the Schwann cells may be stained and the stem fibres situated just beneath the epidermis may appear to be composed of fine neural strands lying in a blue nucleated cytoplasmic reticulum. In certain situations in this cytoplasmic stream, stem fibres of ordinary diameter appear as a series of discontinuous segments lying among a reticulated mass of neurofibrillar-like strands. Such appearances give the impression of a protoplasmically-continuous nerve net lying just beneath the epidermis from which small terminal sprouts arise at intervals (fig. 5, D).

Dendritic cells may be intensely stained in some specimens, particularly in those from man, and terminal nerve fibres may appear to end in specific relationship to them or even to fuse with their processes. In some specimens nerve fibres may appear to enter and to terminate within the cytoplasm of the epidermal or 'dendritic' cells, whilst in other specimens, or in other regions of the same specimen, the terminal filaments all appear to be situated extracellularly (fig. 5, E).

(*b*) *Nerves ending in the dermis.* As in the case of the epidermis, practice and strict attention to detail makes it possible for specimens in which the neural elements resemble those seen following treatment with hyaluronidase to be seen more often. Apart from their location, the stem fibres and their terminals do not differ in appearance from those ending in relation to the epidermis. In other words, their appearance varies from place to place in the same specimen and from specimen to specimen. The same range of appearance is encountered; from stem fibres giving rise to series of fine, independent, freely ending axoplasmic filaments, on the one hand, and through terminal nets to a syncytial network of Schwann cells, in which are embedded fine neural strands, on the other.

2. *Nerves ending among the tissues of hair follicles*

We have found it very difficult to stain nerve terminals related to hair follicles. Stem fibres can be stained regularly and with comparative ease, especially in relation to large hairs, but the fine axoplasmic filaments which spring from the stem fibres are only seen occasionally in the form in which they appear after treatment with hyaluronidase.

In untreated specimens the stem fibres appear to end abruptly in swellings, in the form of fibrillary nets which fuse with their neighbours, or in fibrillae which gradually become attenuated until they are lost to view. The circular series of axons, however, usually appear to be quite separate from the vertical series, although axons within either set alone occasionally appear to fuse with one another.

3. *Encapsulated nerve-endings*

Encapsulated nerve-endings are usually fairly readily stained and their appearance is very striking. On the other hand, careful analysis of the pictures

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FIG. 5 (plate). A–H are from methylene blue preparations. I–O are from silver preparations.

A, shows distorted axis cylinders lying just beneath the epidermis in skin from the human forearm.

B, shows what appears to be a nerve net lying just beneath the epidermis in skin from the human forearm. In the lower part of the picture is a spray of axoplasmic filaments apparently ending in large blobs.

C, shows an irregular mass of terminal axis cylinder elements situated just beneath the epidermis in a human forearm. These resemble the artifacts which can be seen developing under phase-contrast conditions when strong formalin is used to fix a fresh specimen of cornea.

D, shows fragmented axis cylinder substance enclosed within Schwann cell cytoplasm. This was lying just beneath the epidermis in a rabbit ear.

E, shows stained processes of 'dendritic cells' resembling axoplasmic filaments in the epidermis of a rabbit ear.

F, is from a human finger pad and shows distorted stem fibres apparently ending in leaf-like expansions within a capsule. One fine freely ending axoplasmic filament is visible.

G, is from skin of the lower part of a human forearm and shows a distorted stem fibre apparently ending in leaf-like expansions within a capsule.

H, is also from the lower part of the human forearm and shows a stem fibre ending in a mass of fine axoplasmic filaments along the course of which there are at intervals large irregular swellings. No form of capsule is visible.

I, is from a human clitoris and shows axis cylinders breaking up into 'neurofibrillae', and apparently lying in close relation to the nuclei of epidermal cells.

K, is from a human lip and shows a distorted nerve fibre arborizing to give rise to what appears to be a neurofibrillar reticulum in the outer root sheath of a hair. A 'reticulum' derived from another stem axon is clearly seen above the arborizing axis cylinder. The outline of the cell bodies cannot be seen.

L, is from a human clitoris and shows a stem fibre giving rise to what appears to be a neurofibrillary reticulum.

M, is also from a human clitoris and shows a dermal capillary lying close to a number of fine 'neurofibrillary strands'.

N, is from a human finger pad and shows a stem fibre tapering down to end in a fine encapsulated mass of 'terminal reticulum'.

O, is also from a human finger pad and shows a fine axoplasmic filament piercing the capsule and ending in a small knob in the extracapsular tissues.

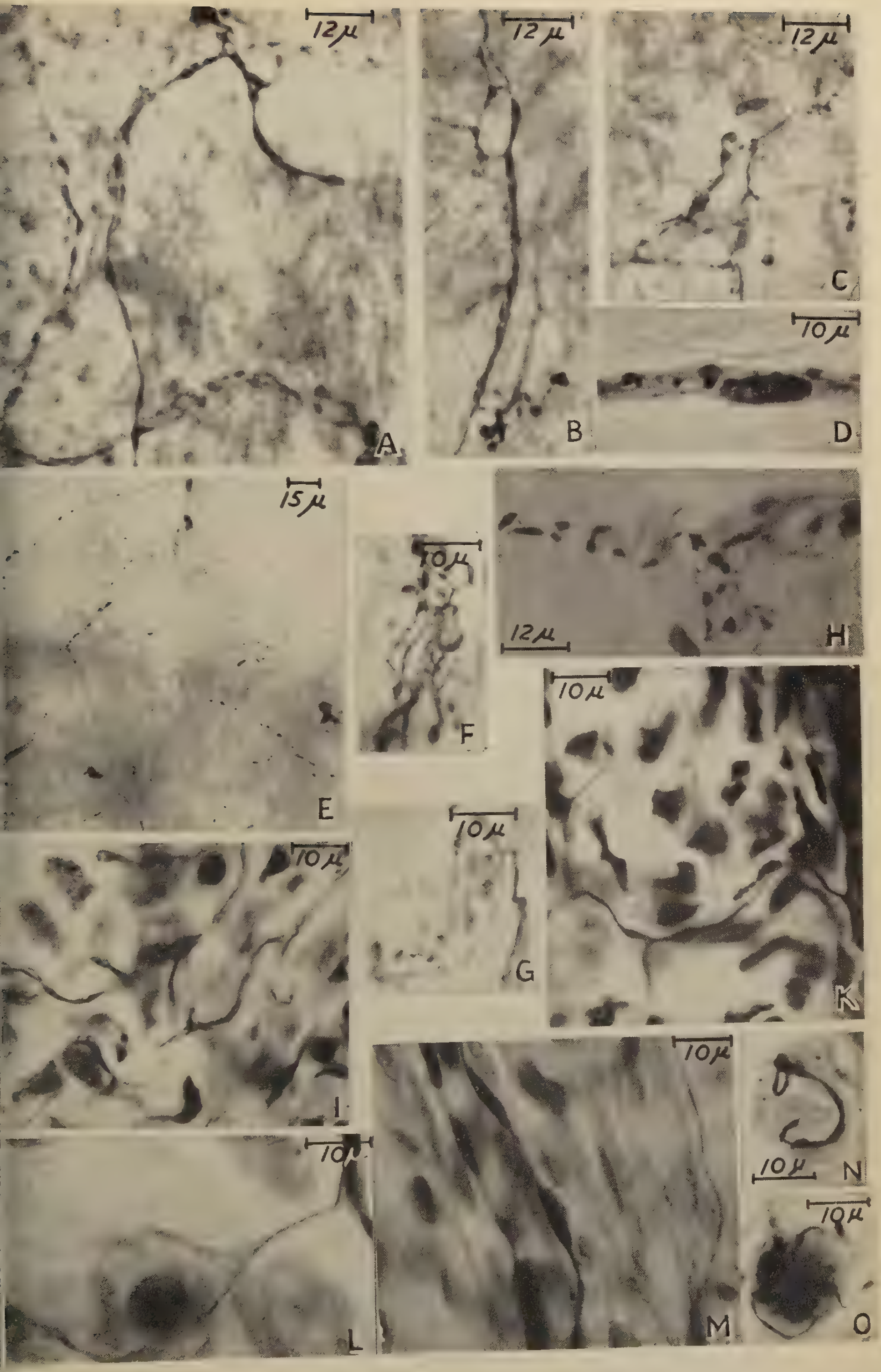

FIG. 5

G. WEDDELL, W. PALLIE, *and* E. PALMER

obtained shows that it is chiefly the stem fibres which are stained. They often vary considerably in diameter in their intra-capsular course which may be relatively simple or extremely tortuous; in addition, they may branch extensively or not at all. The stem fibres terminate in a variety of ways. In solid expansions, which may be spherical or of a crescent shape, in leaf-like expansions, in a mass of fine granules, or in fine, often beaded, fibrillae, which gradually grow finer until they are lost to view (fig. 5, F, G, and H). Encapsulated nerve-endings may be subserved by more than one stem fibre and on occasions stem fibres leave the capsule to end elsewhere in the skin.

We have only occasionally seen encapsulated nerve-endings in which the stem fibres all terminate in a series of fine naked axoplasmic filaments. Indeed, such appearances lead one to suppose that one is observing an unencapsulated nerve-ending, particularly in a specimen in which the nerve fibres are well (i.e. specifically) stained, for the capsular cells are not visible.

B. *Silver impregnation*

1. *Unencapsulated nerve-endings*

(*a*) *Nerves ending in relation to the epidermis.* It is very difficult to find nerve fibres ending in the epidermis. They can be seen in a few rare specimens but even then not many are to be seen and the relationship of the nerve fibres to the cells of the epidermis is very difficult to ascertain, for it varies from specimen to specimen. In one case they appear to lie within the cell, in another they lie extra-cellularly. The nerve fibres commonly fuse with one another.

Our best specimens were prepared by the method of Bielchowsky–Gros as practised by Stöhr (1950). In these specimens more nerve fibres are seen than in any others, but the boundary membranes of the cells comprising the epidermis are impossible to see when the nerve fibres are finest and most abundant (fig. 5, I).

(*b*) *Nerves ending in the dermis.* Unencapsulated nerve-endings in the dermis are rare following silver impregnation, unless the methods advocated by Stöhr are used. In such specimens, cell boundaries are difficult to distinguish. Occasionally, fine, apparently naked nerve filaments are seen wandering among the tissues of the dermis in relatively close relationship (fig. 5, M) to capillaries, but they always appear to terminate by attenuation. The commonest picture is similar to that seen after overstaining with methylene blue. It consists of a rich network of neural filaments, which are often beaded, lying in strands which form a continuous network in the dermis, being most dense where the capillaries are most abundant. A few fibrillae appear to end freely but they mostly fuse with one another. At intervals, stem fibres can be seen giving rise to what appear to be neurofibrillae (fig. 5, L).

2. *Nerves ending in the tissues of hair follicles*

Stem fibres are relatively easy to impregnate, although they appear to be somewhat larger in diameter than when stained with methylene blue. The axoplasmic filaments, however, are very hard to impregnate without causing swelling and fragmentation of the stem fibre. The terminal filaments when impregnated either appear to grow finer and finer until they are lost to view, or they end in some form of expansion which may be solid and disk-like, or a reticulate formation of fine fibrillary strands. The filaments often appear to fuse with one another. As in the case of methylene blue preparations, however, filaments in the outer root sheath always remain separate from those lying in close relation to the vitreous layer. Very few filaments are seen within the vitreous layer among the cells of the outer root sheath, although they have been found in a few preparations. The relationship of the filaments and their terminals to the cytoplasm of the various cells in the follicle varies. In some cases they seem to be within the cytoplasm, in other instances on the cell surface (fig. 5, K).

3. *Encapsulated nerve-endings*

These are easy to distinguish when they are impregnated, although they cannot be impregnated with certainty in any given piece of tissue. The morphology of the tortuous stem fibre or fibres and the bulbous ring and leaf-like terminals to which it gives rise and which lie within a faintly impregnated capsule of cells is striking. Axoplasmic filaments springing from the stem fibres are seen within the capsule in some specimens but not in others. They vary in thickness and appear to terminate either by fusing with their neighbours, or freely in some form of expansion. The relationship of the terminal expansion to the capsular cells is impossible to determine with any certainty. The silver methods advocated by Stöhr and his pupils come closest to representing stem fibres and axoplasmic filamentous terminals in material not treated with hyaluronidase (fig. 5, N and O).

DISCUSSION

We have demonstrated that all the cutaneous nerves in the mammalian skin which we have examined end in terminal arborizations of fine naked axoplasmic filaments. Each set of filaments is derived from an ensheathed stem fibre and each filament ends freely in an extra-cellular position. The terminal arborizations arise from stem fibres of varying diameter and are disposed in different positions within the skin and in relation to different tissue elements among which they lie. Thus the actual terminal filaments within the encapsulated nerve-endings appear in no way different from those in the epidermis or those ending in the tissues of a hair follicle. In *this* respect, therefore, the termination of nerve fibres in mammalian skin does not differ from the termination of nerve fibres in the cornea of numerous species (Zander and Weddell, 1951) or even, according to Whitear (1952), from the termination of nerve fibres in the skin of Teleost fishes.

The pictures obtained without the use of hyaluronidase resemble those obtained by Weddell and Zander (1950) in the cornea when it was roughly andled, when it was exposed to high concentrations of methylene blue, or hen it was fixed in formalin. In other words, they contain numerous artifacts hich can be accounted for (Weddell and Pallie, 1954) on the basis that a umber of nerve fibres and particularly the fine axoplasmic filaments have een damaged and distorted in the course of handling, fixation, and staining, nd that the dye or impregnating agent did not reach all the nerve fibres and erminals evenly at the same time and in the same concentration. Indeed, Veddell, Pallie, and Palmer (1954) have shown that, if allowance is made for rtifacts, the observations which have been reported in the literature resemble losely those which we have made following the use of hyaluronidase.

It is therefore from a neurohistological arrangement of the kind we have escribed in this paper that appropriate stimuli in man give rise to the percepions embodied in the phrase 'cutaneous sensibility'. In two previous publicaions (Sinclair, Weddell, and Zander, 1952; Hagen, Knoche, Sinclair, and Veddell, 1953) it has been shown that the four commonly recognized modaliies of cutaneous sensibility (touch, cold, warmth, and pain) can all be aroused rom the front of the forearm and the back of the ear (hairy skin) and from the inger pads and mucous membrane of the lip (glabrous skin) with almost equal ase in spite of certain obvious yet gross differences in their neurohistological onfigurations. In the hairy skin, numerous unencapsulated nerve-endings are ound together with terminals concentrated in the tissues of hair follicles but here are no encapsulated nerve-endings. In glabrous skin there are also umerous unencapsulated nerve-endings together with a large number of encapsulated nerve-endings of such diverse form that they cannot be rigidly classified on a morphological basis.

These observations demonstrated that, in hairy skin, cold sensibility cannot be related to encapsulated nerve-endings and, further, in glabrous skin, cold sensibility cannot be related to a specified encapsulated nerve-ending (Krause end-bulb) as has been suggested by a number of observers (Bazett, McGlone, Williams, and Lufkin, 1932).

Our present observations take us a step further. We can now suggest that nerve-terminals as such can only be specifically related to sensory modalities in respect of their situation in the skin relative to the stem fibres from which they arise, bearing in mind the influence of their chemical environment.

For instance, in the case of encapsulated nerve-endings the numerous naked axoplasmic filaments are concentrated in a small space. They are short and, broadly speaking, arranged in circumferential planes in a sphere of cells lying approximately at right angles to the stem fibres from which they arise. This arrangement is such that the slightest degree of deformation will affect the relationship of some of the axoplasmic filaments to the stem fibres from which they arise. The same kind of effect occurs in hair follicles but here the axoplasmic filaments are arranged around the hair in two separate, closely concentrated groups; one group lies parallel to the hair and within the vitreous membrane and the other encircles the hair outside the membrane. Again, deformation by bending, pulling, or pushing the hair will alter the relationship of the axoplasmic filaments to the stem fibres from which they arise. Hence i is logical to correlate touch sensibility with encapsulated nerve-endings, and with hairs.

On the other hand, small degrees of local deformation are less likely to create sufficient local activity in widespread arborizations of free terminals to activate stem fibres, unless both terminals and stem fibres are situated comparatively superficially in the skin. It will be recalled that Waterston (1923) adduced evidence in support of the contention that the most superficially situated free nerve-terminals subserved touch sensibility. Our observations are not at variance with this suggestion but, if this is so, then some of these or the remaining free terminals in the dermis must, at least in hairy skin, subserve temperature sensibility. It is of interest that some of these terminals are situated among the arterioles, arteriovenous anastomoses, and capillaries of the skin, positions highly suitable for thermometric purposes.

It now becomes much more difficult to correlate pain indiscriminately with free nerve-terminals (Woollard, Weddell, and Harpman, 1940), although the evidence that some correlation exists between pain and free terminals is overwhelming (Weddell, 1941, 1945; Weddell and Sinclair, 1953).

Apart from their possible relationships to specific sensory modalities, there is now evidence of a physiological nature which suggests that naked nerve-terminals have properties and therefore functions which are different from those of the stem or conducting fibres. The morphology and relationships of many of the free nerve-terminals suggests that it is unlikely that a propagated disturbance will pass along every axoplasmic filament, wherever it is situated, every time it is stimulated; local non-propagated disturbances are much more likely to be encountered if the stimulus is anywhere near threshold. The work of Alvarez-Buylla and Ramirez de Arellano (1953) and Gray and Sato (1953) on the Pacinian corpuscle, in which the stem fibre is known to give rise to and to terminate in a complicated series of fine axoplasmic extensions which end in the wall of the corpuscle, has shown that this is true in the case of an encapsulated nerve-ending. They showed that mechanical stimuli of low amplitude evoked no response in the parent nerve fibre of a Pacinian corpuscle. When the amplitude was increased, however, a local potential was recorded up to 4 mm. from the corpuscle. The amplitude of the local potential increased smoothly with the amplitude of the mechanical stimulus until at threshold a propagated disturbance emerged from the late part of the local potential.

If it is accepted that arborizations of free nerve-terminals in the skin also respond to stimulation by giving rise to local non-propagated impulses, then a sufficient force in space and in time might activate enough of the terminals of a given stem fibre to cause it to discharge. In other words, there should be evidence of spatial and temporal summation of stimuli in the skin.

On the basis of the above argument, the all-or-nothing law in relation to cutaneous sensibility is only applicable when the stem or conducting nerve fibre is involved. Most relevant stimuli received in the course of everyday life are, of course, gross enough to ensure that certain terminals (in particular, hose around hairs) behave as though they were merely an extension of the onducting fibre and an all-or-nothing response does occur. However, when hresholds and reaction times or the effect of environmental changes on ensory perception are under consideration, it may be necessary to think in erms other than of the all-or-nothing principle and the work of Lele, Sinclair, nd Weddell (1953) on reaction time to touch has shown that there is a large scatter in reaction times and that spatial summation does occur.

The existence of a 'nocifensor system' of nerve fibres postulated by Lewis has been the subject of doubt, for it has been said that it cannot be distinguished as a separate anatomical entity (Woollard and others, 1940; Walshe, 1942; Weddell, 1945). It was suggested by Woollard and others (1940) and again by Weddell (1945) that 'nocifensor reactions' could be identified with the terminal system subserving 'pain', for in a piece of skin from which pain only could be aroused together with nocifensor reactions, only one stem fibre and set of nerve terminals could be demonstrated. Nevertheless, Lewis has shown that flare can be evoked apart from pain. Careful re-evaluation of the work of Lewis (1942) in the light of our present observations suggests that some of the free axoplasmic filaments in the skin may take part in a series of peripheral physico-chemical reactions without initiating impulses in the ensheathed stem axons from which they are derived, provided that the stimulus is not excessive and that the stimulating agent is confined to a relatively limited layer in the skin. Some of the arborizations which we have seen are quite extensive enough for such a suggestion to be acceptable.

There are two additional pieces of evidence which support this suggestion. In the first place, Lewis classified hyperalgesia (in the sense of ability to arouse pain by brushing) as a nocifensor reaction; now, if our suggestion as to the mechanism of flare is correct, then additional stimuli, far below the normal threshold, might be expected to increase the local response sufficiently to fire the stem fibres and so to give rise to pain.

In the second place, hyperalgesia is known to occur in the marginal zone of a freshly denervated area of skin some 48 hours after operation, after which it passes off (Trotter and Davies, 1913). In 1948 Weddell, Sinclair, and Feindel found that the neurohistological picture beneath such a zone consisted of fine short naked axoplasmic sprouts derived from the surrounding normal nerve-fibres which were growing towards the denervated area. The terminals were only a short distance from their stem fibres and presumably a local response evoked by a minimal stimulus would fire the parent fibre. The fact that the hyperalgesia passes off is explained by the observation that the sprouts continue to advance towards the denervated zone (Weddell, Guttmann, and Gutman, 1941) for some weeks, thus increasing their distance from the stem fibres from which they arise.

As the result of the present investigation, it is perhaps permissible to reconsider the position of the neuron as a morphological and functional unit. Centrally, it is well recognized that afferent cutaneous nerve fibres make synaptic connexions by flowering into a series of fine axoplasmic extensions which are so similar to those in the periphery that one may regard them a identical from the morphological point of view. For the purposes of functiona interpretation therefore, it seems legitimate to consider cutaneous sensory neurons as being made up of three parts: (1) a peripheral spray of naked filamentous axoplasmic extensions of varying length, profusion, and localization (2) an ensheathed axon of a larger diameter which may or may not be visibly myelinated; and (3) a central spray of fine naked axoplasmic extensions relate synaptically to other neurons within the central nervous system.

Katz (1952) has recently emphasized the importance of local non-explosive responses and has stated, in relation to sensory perception, 'that what we fee about a sensory stimulus is a more faithful image of the stimulus itself than o the nerve impulses which helped to report it'. The morphological picture which we have painted seems to make a suitable background for the physiological observations which led Katz to make this statement.

This work was made possible by a grant from the Rockefeller Foundation which is gratefully acknowledged. Our thanks are due to Dr. C. F. A. Pantin, F.R.S., for a number of helpful suggestions, and we would also like to thank Miss Jean Gurden and Mr. Frank Blackwell for their skilled technical assistance.

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Relations between Metachronism and the Direction of Ciliary Beat in Metazoa

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With one plate (fig. 2)

SUMMARY

In various ciliated epithelia one can distinguish either symplectic, antiplectic, dexioplectic, or laeoplectic metachronal waves, according to whether the effective beat of their cilia is with, against, to the right of, or to the left of, the direction of movement of the waves. In a given epithelium the relation between the direction of the waves and the direction of beat is constant.

Symplectic waves are probably associated with the transport of large particles, or viscous masses of mucus; antiplectic and diaplectic waves are better suited for creating water currents. It is unusual to find both dexioplectic and laeoplectic waves in the same animal; indeed, it is the rule for only one of these two types of metachronism to occur throughout a given systematic group. Dexioplectic waves are found in Protochordata, primitive Spiralia, the velum of Eulamellibranchiata, Phoronidea, Brachiopoda, Bdelloidea, and Melicertida; laeoplectic waves are found in *Chaetopterus*, Mollusca generally, Bryozoa, and Ploima. The incidence of dexioplectic and laeoplectic metachronism is not influenced by morphological reversals of symmetry such as the *situs inversus*. The relation between the direction of beat and the direction of the waves is therefore probably determined by cytological or stereochemical factors, rather than by gross morphology.

CONTENTS

	PAGE
INTRODUCTION, INCLUDING DEFINITIONS OF TERMS USED	504
SYMPLECTIC AND ANTIPLECTIC METACHRONISM	504
DIAPLECTIC METACHRONISM	505
PRELIMINARY SURVEY OF THE DISTRIBUTION OF DEXIOPLECTIC AND LAEOPLECTIC METACHRONISM IN METAZOA	507
Protochordata	507
Echinodermata	509
Turbellaria	509
Nemertini	510
Sipunculoidea	510
Archiannelida	510
Polychaeta	510
Mollusca	511
Phoronidea	514
Brachiopoda	515
Bryozoa	515
Rotifera	515
CONCLUSIONS OF SYSTEMATIC INTEREST	515
PROBABLE LOCATION OF THE FACTOR DETERMINING WHETHER DIAPLECTIC METACHRONISM IS DEXIO- OR LAEOPLECTIC	516
TABLES	517
REFERENCES	520

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INTRODUCTION, INCLUDING DEFINITIONS OF TERMS USED

IN most ciliated epithelia the cilia are arranged in rows, in two directions which are at right angles to one another. One of these directions approximates to the direction in which the cilia beat. The rows which run in this direction may therefore be called orthoplectic rows, and those which run in the other direction, diaplectic rows. In one of these two directions the cilia of a given row often beat synchronously, but in the other direction each cilium beats immediately after its neighbour, producing metachronal waves.

In the best-known examples of ciliary metachronism the waves move along the orthoplectic rows of cilia, travelling either in the same direction as the effective beat (these may be termed symplectic waves) or in the opposite direction (antiplectic waves). In many animals, however, the waves move along the diaplectic rows, and the effective beat may then be either to the right or to the left of the direction of movement of the waves, which may therefore be termed dexioplectic or laeoplectic waves.

It is well known, from observations on orthoplectic waves, that the relation between the direction of metachronism and the direction of beat is constant in a given epithelium. This constant relation is maintained even when the usual direction of beat is reversed, whether teratologically as in the gills of *Mytilus* (Atkins, 1930), surgically as in the epithelium of the frog's mouth (Brücke, 1916), in response to chemical stimulation, as in the labial cilia of *Metridium* (Parker, 1905), or voluntarily as in the ctenophores responding to mechanical stimuli (Fedele, 1925), and in the enteropneust, *Saccoglossus cambrensis*, when it reverses the beat of the trunk cilia. Thus in a variety of epithelia, showing both symplectic and antiplectic waves, whenever the ciliary beat is reversed the direction of movement of the waves is reversed too. There seems to be some link between the direction of the effective beat and the direction of movement of the waves.

The constancy of this link would be less surprising were it always to have adaptive value, but though there are functional differences between symplectic and antiplectic metachronism, there are no such differences between dexio- and laeoplectic metachronism. The incidence of one or the other of the two types of diaplectic metachronism must therefore, in general, have purely phylogenetic significance.

SYMPLECTIC AND ANTIPLECTIC METACHRONISM

Symplectic metachronism is particularly well known from early observations on the cilia of the frog's gullet and from the analogy of wind passing over a field of corn. Nevertheless, it occurs comparatively infrequently, and was not observed during the present survey, which mainly involved marine animals. This type of metachronal co-ordination requires the cilia to be bunched together during their effective stroke (fig. 1), an arrangement which would be well adapted for the transport of large particles or viscous masses of mucus. If a cilium should be checked by contact with a large particle, its neighbours, following behind it, would immediately catch up with it and help it to move the particle along.

This arrangement would not be the most efficient way of causing a current in a fluid medium of low viscosity, since each cilium would push water already set in motion by the one behind. Antiplectic metachronism, which may be seen in a variety of animals (table 1, see p. 517), does this more efficiently, since it leads to the cilia, which are performing their effective stroke, being spaced out like the blades of a paddle wheel, whilst those which are recovering present the least possible resistance to water flow by being bunched together (fig. 1). This consideration raises the question whether the reversal of the effective beat in, for instance, Ctenophora, inevitably follows reversal of metachronism, without any other change taking place. In many ciliated epithelia, however, it can be seen clearly that the flexural characteristics of the cilia are of major importance in determining the direction of beat.

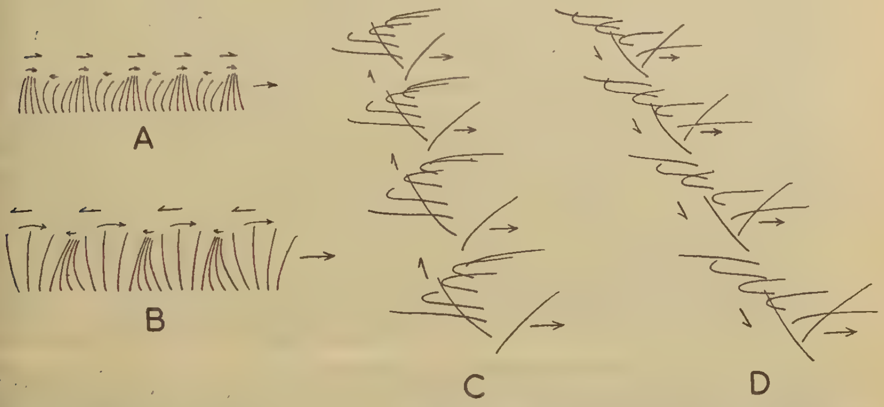

FIG. 1. Diagrams to illustrate the arrangement of cilia which are subject to the four main types of metachronal co-ordination. Each diagram represents a row of cilia the effective stroke of which is to the right and approximately in the plane of the paper (the diaplectic rows *C* and *D* are tilted to give a stereoscopic view along them). In this, and in subsequent figures, the direction of movement of metachronal waves is shown by single-barbed arrows, and the direction of effective beat by complete arrows. A, symplectic metachronism, in which the cilia performing the effective stroke are bunched together; B, antiplectic metachronism, in which the cilia performing the effective stroke are spread out; C, dexioplectic, and D, laeoplectic metachronism.

DIAPLECTIC METACHRONISM

For the sake of clarity, diagrams of the two types of diaplectic metachronism are given in fig. 1. Diaplectic waves are better illustrated in Dr. D. P. Wilson's photographs (fig. 2) which greatly aid analysis of their form. The ciliated girdles of larvae (fig. 4) and the lateral belts of cilia, which fringe branchial and lophophoral filaments and gill slits in a variety of adult animals, provide familiar examples of this type of metachronism, which is therefore found generally, but not exclusively, wherever narrow belts of cilia are elongated transversely to the direction of beat. The cilia are often large, and the metachronal waves particularly conspicuous.

A functional advantage of diaplectic metachronism is that it allows large, strongly flexural cilia to operate freely, without striking their neighbours, since the beat is synchronized throughout each orthoplectic row. Cilia out of phase in the direction of beat must either move with a small amplitude or be widely spaced (as in ctenophores). Another result of this extra freedom is that cilia showing diaplectic metachronism may undergo fusion between adjacent individuals not only within the diaplectic rows, as in the undulating membranes of many ciliates, but alternatively, within the orthoplectic rows, as in the compound cilia of the nudibranch velum (Carter, 1928). On the other hand, fusion of cilia showing orthoplectic metachronism is only possible within the diaplectic rows, as in the swimming plates of ctenophores.

Since it would be inefficient to have cilia of more than a certain size beating in crowded orthoplectic rows, and therefore moving in water already set in motion by their neighbours, the number of cilia in each orthoplectic row is often small, or unity (fig. 7, p. 512). The narrow diaplectic belts, which result from such an arrangement, often occur repeatedly, at intervals, in a manner which may or may not be related to metamerism of the animal (e.g. figs. 5, A and 6, D, pp. 509 and 511).

The direction of rotation of certain ciliated larvae indicates that the beat of the cilia is not always exactly at right angles to the direction of movement of the metachronal waves. Most larvae with diaplectic ciliary girdles rotate constantly in one direction, and this is generally opposite to the direction of movement of the waves. The speed of rotation is usually much less than the speed of the waves, but larvae of a spherical or saucer-like form, with little resistance to rotation, rotate at a speed nearly as great as that of the waves, which suggests that the waves are responsible for the rotation. This is to be expected (Burdon-Jones, 1952), for each group of cilia on the recovery stroke

---

FIG. 2 (plate). A, tornaria larva of *Glossobalanus sarniensis*, viewed abapically (with the telotroch beating towards the observer). The metachronal waves, being dexioplectic, appear, from this viewpoint, to move clockwise. The prominent cilia, which form the crests of the waves, are those performing their effective stroke. Immediately clockwise from these, in the troughs of the waves, the cilia are extended away from the observer, at the beginning of the effective stroke. Immediately anticlockwise from the crests of the waves are cilia at the end of the effective stroke. These do not lie flat, so do not form troughs. Further anticlockwise, between these and the troughs, are cilia on the recovery stroke, which reflect light strongly, presumably because they are strongly flexed.

B, the same, viewed ventrolaterally, with the telotrochal waves of the near side moving from left to right of the observer. In this view the crests of the waves are formed by the cilia at the beginning of the effective stroke. Immediately to the left of these the cilia are performing the effective stroke, the rapidity of which is reflected in the steepness of this part of the waves. To the left of the troughs lie the cilia which are on the recovery stroke. The oblique arrangement of these cilia, as the animal moves through the water, is probably responsible for its rotation.

(*Photographed by Dr. D. P. Wilson, by electronic flash.*)

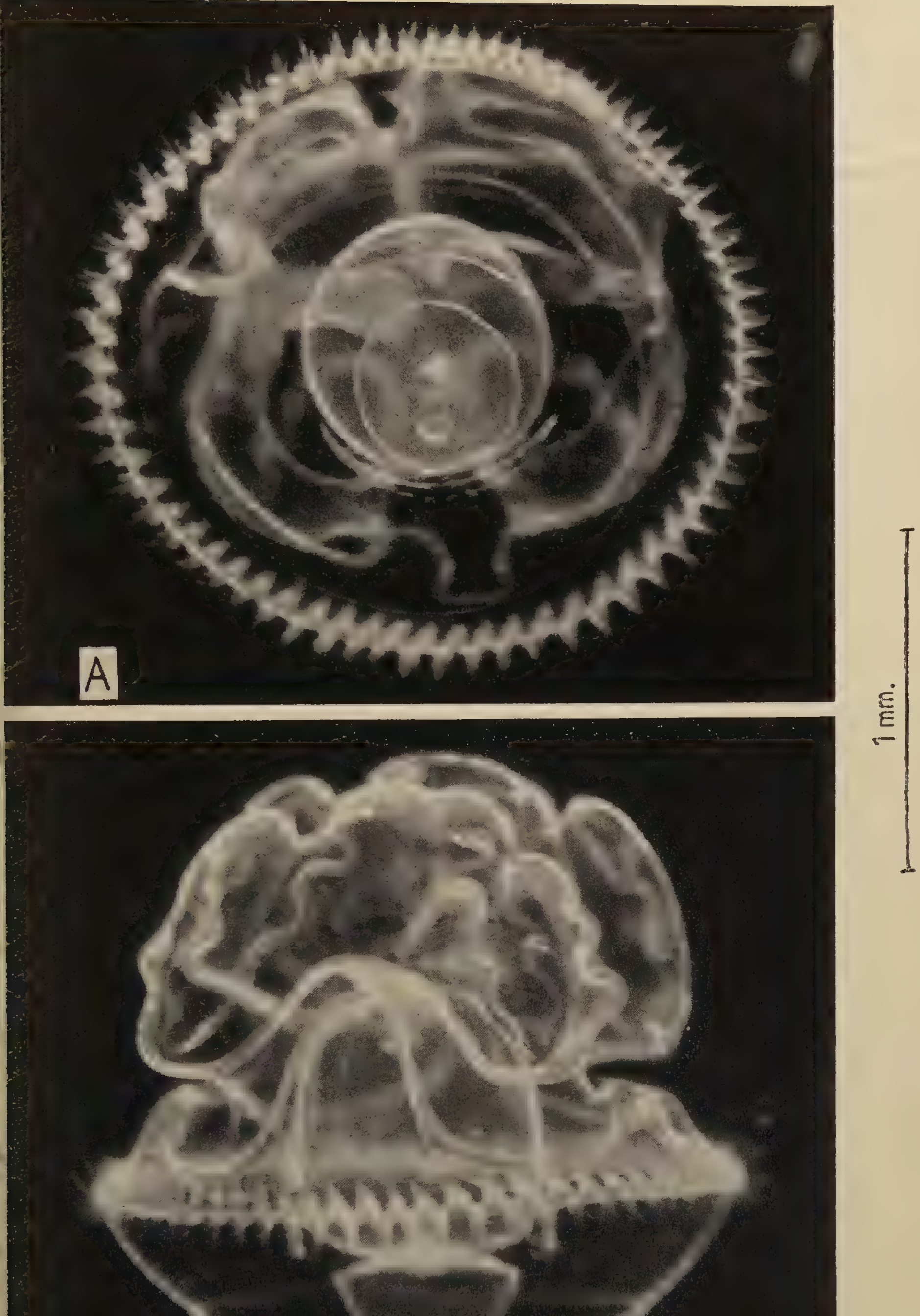

Fig. 2

KNIGHT-JONES

is arranged so that it presents an oblique (though of course incomplete) surface to the water through which the larva is moving (fig. 2, B). In many molluscs, however, the veligers rotate constantly in the same direction as that in which the waves move, which suggests that the beat of their cilia is somewhat oblique. It should be noted, too, that the lateral cilia of the lamellibranch ctenidia have obliquely arranged basal granules (Atkins, 1938).

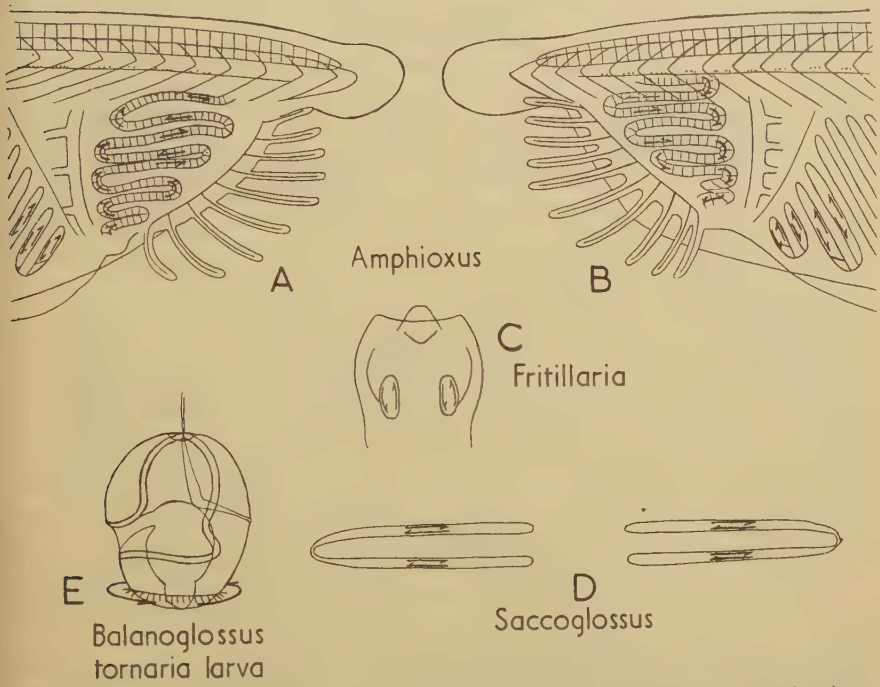

FIG. 3. Directions of movement of dexioplectic metachronal waves in Protochordata. A, anterior end of *Amphioxus*, from right side, showing waves of wheel organ and gill slits; B, the same, from left side; C, gill slits of *Fritillaria*, viewed ventrally (with cilia beating towards observer), after Fol; D, gill slits of *Saccoglossus*, viewed from inside pharynx (with cilia beating away from observer); E, tornaria of *Balanoglossus*, showing waves of telotroch.

PRELIMINARY SURVEY OF THE DISTRIBUTION OF DEXIOPLECTIC AND LAEOPLECTIC METACHRONISM IN METAZOA

Many of the following observations relate to planktonic larvae (e.g. fig. 4), which were reared from spawn or from artificial fertilizations, or which were identified from the plankton at Naples or at Bangor.

Protochordata (table 2, p. 517)

Wherever diaplectic waves occur in this group they invariably beat to the right. The waves produced by the cilia bordering the gill slits therefore move in what appears, from outside, to be an anticlockwise direction on both sides of the body (e.g. fig. 3, C). This bilaterally asymmetrical movement previously seemed difficult to explain (Knight-Jones and Millar, 1949), but it is of a sort found, as a rule, wherever diaplectic metachronism occurs. Similarly, the waves of the wheel organ in *Amphioxus* travel dorsally on the right side and ventrally on the left (fig. 3, A and B), the beat of the cilia being in a generally posterior direction, at right angles to the winding course of the ciliated tract. The waves of the telotroch in larval Enteropneusta (fig. 2) are also dexioplectic.

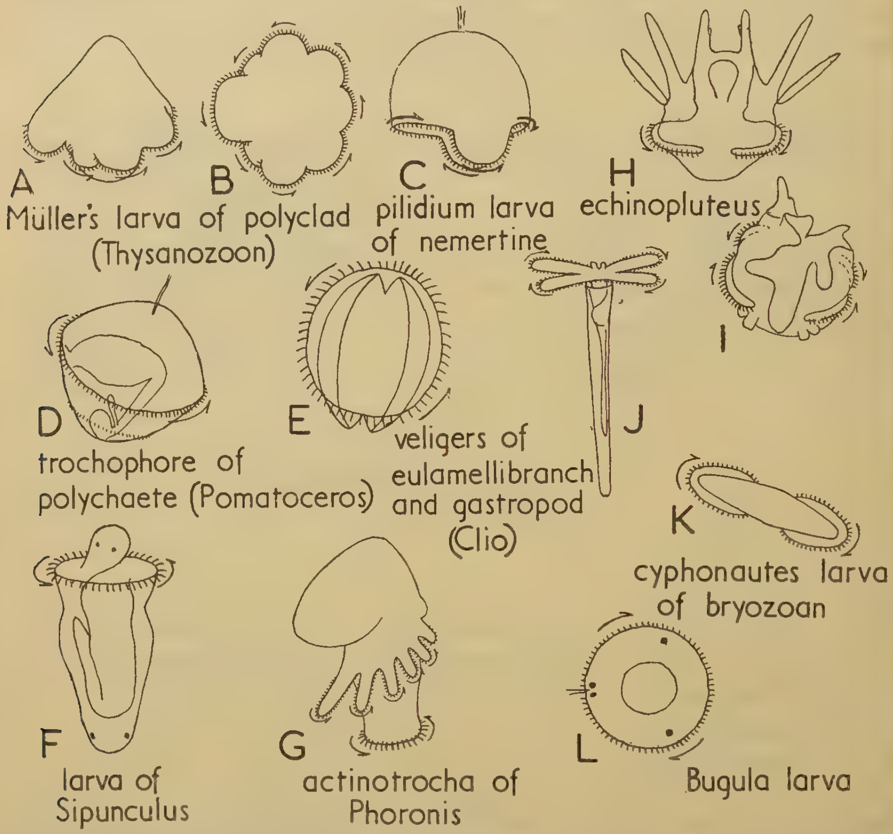
A
B
Müller's larva of polyclad (Thysanozoon)
C
pilidium larva of nemertine
H
echinopluteus
I
D
trochophore of polychaete (Pomatoceros)
E
veligers of eulamellibranch and gastropod (Clio)
J
K
cyphonautes larva of bryozoan
F
larva of Sipunculus
G
actinotrocha of Phoronis
L
Bugula larva

FIG. 4. Movement of diaplectic metachronal waves round the ciliated girdles of some marine invertebrate larvae. The cilia of all beat posteriorly or abapically (directly or obliquely away from the observer in all these figures except A and C). In A to G the waves are dexioplectic; in H and I the waves move bilaterally symmetrically, being dexioplectic on one side and laeoplectic on the other; in J to L the waves are laeoplectic. A, Müller's larva of *Thysanozoon*, from left side; B, the same, viewed apically; C, pilidium larva of nemertine, from left side; D, trochophore of *Pomatoceros*, apico-lateral view, from left side; E, veliger of eulamellibranch, viewed apically; F, larva of *Sipunculus*, apico-lateral view, from right side; G, actinotroch of *Phoronis*, from left side; H, echinopluteus, viewed ventrally; I, echinopluteus about to metamorphose, apico-lateral view, from right side; J, veliger of the pteropod, *Clio* (*Creseis*) *acicula*, viewed apico-ventrally; K, cyphonautes larva of bryozoan, viewed apically; L, larva of *Bugula*, viewed apically.

Echinodermata

The echinopluteus (fig. 4, D), with its powerfully ciliated epaulettes, is the only animal observed which has achieved a symmetrical arrangement of diaplectic metachronism, by having dexioplectic waves on one side and laeoplectic waves on the other. This arrangement of opposing waves must counter the tendency for the whole body to rotate, which is shown by many larvae with waves which encircle the body always in the same direction. Perhaps rotation would hamper feeding or some other activity of the echinopluteus.

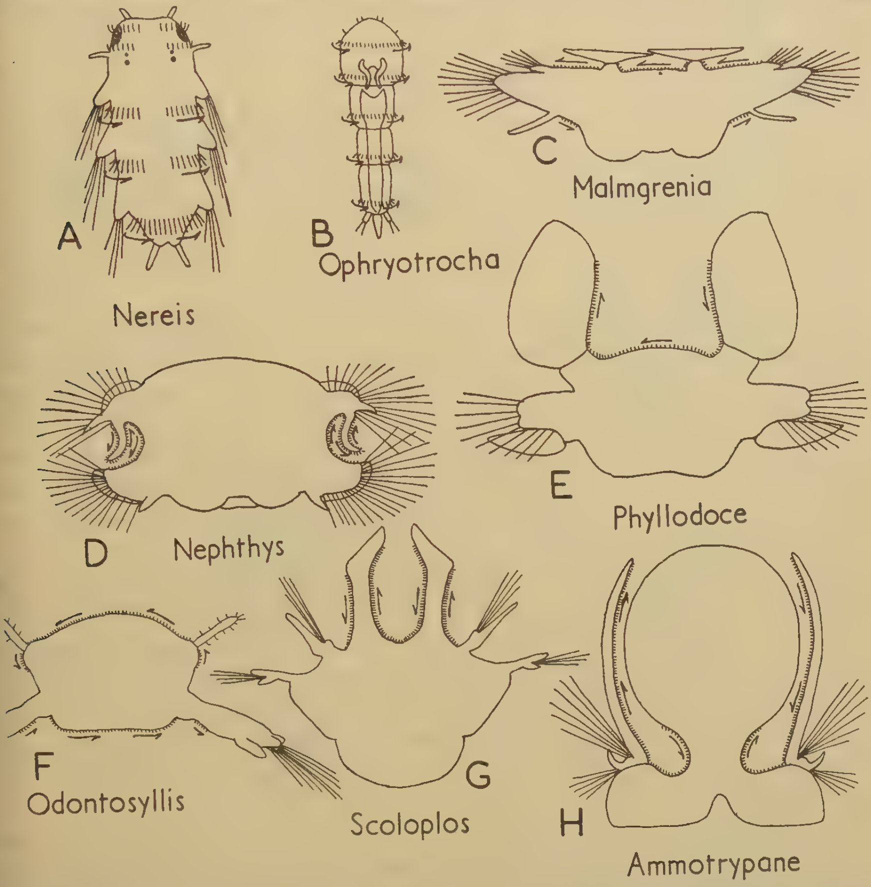

FIG. 5. Movement of the dexioplectic metachronal waves shown by the segmentally arranged transverse ciliated tracts of polychaetes: A and B, larvae of *Nereis pelagica* and *Ophryotrocha puerilis*, in dorsal view. The remaining figures represent transverse sections, viewed anteriorly, with the cilia beating away from the observer: C, *Malmgrenia castanea*; D, *Nephthys caeca*; E, *Phyllodoce mucosa*; F, *Odontosyllis gibba*; G, *Scoloplos armiger*; H, *Ammotrypane aulogaster*.

Turbellaria (table 3, p. 518)

Dexioplectic waves are found on the ciliated lobes of polyclad larvae (fig. 4, B), as originally figured by Müller (1850).

Nemertini

Dexioplectic waves travel round the marginal lappets of pilidium larvae (fig. 4, C).

Sipunculoidea

Dexioplectic waves travel round the ciliated girdle in larvae of *Sipunculus nudus* (fig. 4, F).

Archiannelida

Dexioplectic waves travel round the head of *Dinophilus taeniatus*. The small cilia, which beat posteriorly, were difficult to observe in active entire animals, but they were easily examined in a head which had been partially severed from the remainder of the body.

Polychaeta (table 3, p. 518)

Dexioplectic waves were observed in 56 out of 57 identified larvae and adults, which showed diaplectic metachronism, and in many unidentified larvae. As Segrove (1938) remarked, the ciliated girdles of larval polychaetes have their counterparts in the segmental transverse bands of many adults (fig. 5). These adult cilia are probably respiratory in function and generally beat posteriorly. Many polychaetes have longitudinal rows of cilia along the sides of the body (fig. 6, A and B). In some forms, such as *Syllis prolifera* and *Phyllodoce maculata*, these cilia beat ventrally, so the dexioplectic waves move anteriorly on the right side and posteriorly on the left; but in others, such as *Scalisetosus pellucidus* and *Sabellaria alveolata*, they beat dorsally, so the waves move posteriorly on the right side and anteriorly on the left. Several sedentary polychaetes, such as *Stylarioides eruca* (fig. 6, C and D), *Flabelligera affinis*, *Ampharete grubei*, and *Sabellaria alveolata*, have branchiae with cilia which are arranged in transverse bands and which drive currents distally, and dexioplectic waves which follow each band in a partial or complete circuit of the branchia.

An apparent example of reversal of cilia was observed in *Microspio mecznikowianus*, which lives in sandy mud at Naples. When this form is left undisturbed its branchial cilia beat anteriorly, so their functions probably include the expulsion of waste products, as in many tubicolous forms. But when *Microspio* is agitated these cilia beat posteriorly, as in other Spionidae, drawing fresh water into the tube to meet increased oxygen requirements. Whenever the ciliary current reverses, the metachronal waves also travel in the reverse direction, but close examination of the inner surfaces of the branchiae shows that there are two closely adjacent rows of cilia on each, only one of which is active at a time. The cilia of the row which is active beat over the top of those of the other row, which lie flat, at the beginning of the effective stroke, as resting cilia usually do (Gray, 1928). The metachronal waves of the active cilia are always dexioplectic, those of the two adjacent rows travelling in opposite directions (fig. 7).

The only polychaetes observed with laeoplectic waves were larvae of *Chaetopterus variopedatus*. Adult Chaetopteridae have small cilia, which do not show clearly diaplectic metachronism.

Mollusca (table 4, p. 518)

The lateral cilia of the ctenidia show laeoplectic waves in all classes, except in Cephalopoda, which have unciliated muscular ctenidia expanding and contracting like lungs, and in Scaphopoda, which lack ctenidia. The lateral cilia beat towards the dorsal surface of the ctenidium, drawing water up between the lamellae (fig. 8, B and F). In most lamellibranchs the lamellae are drawn out into filaments, which are kinked to form a V on each side of the ctenidial axis. Since the lateral cilia always beat into the space enclosed by each V, the laeoplectic waves always appear to move as a pencil moves in writing a W, irrespective of whether the observer is viewing the anterior or the posterior surfaces of the filaments (fig. 8, G). The knowledge that the waves are

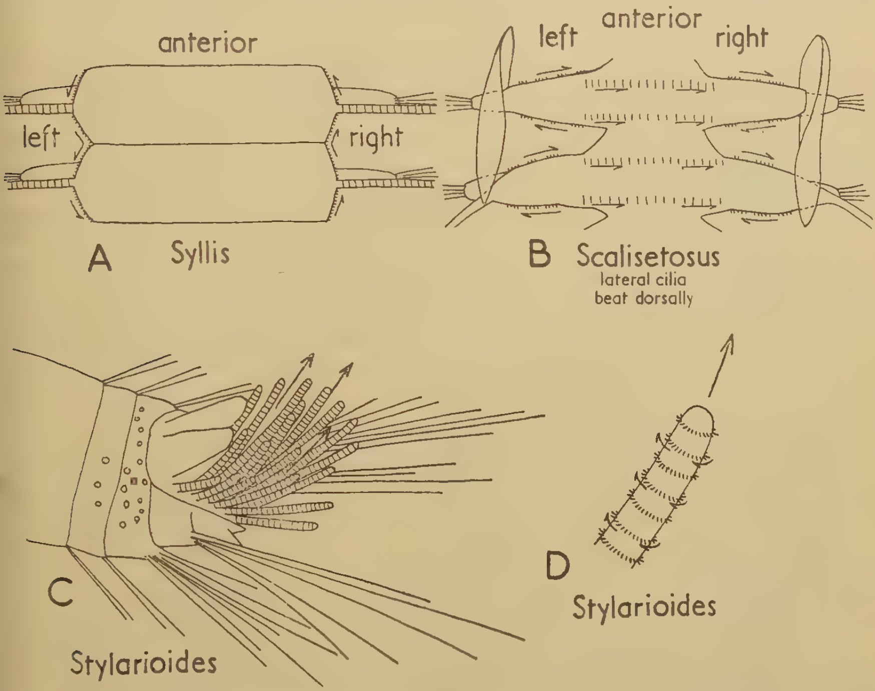

FIG. 6. Further examples of the dexioplectic metachronal waves of polychaetes. A, two segments of *Syllis prolifera*, viewed dorsally, with the longitudinal ciliated tracts of each side beating away from the observer; B, two segments of *Scalisetosus pellucidus*, viewed dorsally, with the longitudinal ciliated tracts of each side beating towards the observer, and the transverse tracts of the dorsal surface beating posteriorly (towards the bottom of the figure); C, anterior end of *Stylarioides eruca*, viewed dorso-laterally from the right side, showing the currents produced by the branchial cilia, which beat distally; D, distal end of a branchia from *Stylarioides*, showing the encircling rings of cilia, the current produced, and the direction of the metachronal waves.

laeoplectic helps the study of ctenidial ontogenesis. In small post-larvae it is not easy to see which way the cilia of the first gill filaments are beating, but the distinct diaplectic waves, which are characteristic of the lateral cilia, can easily be seen through the transparent shells (fig. 8, H and I). On the justifiable

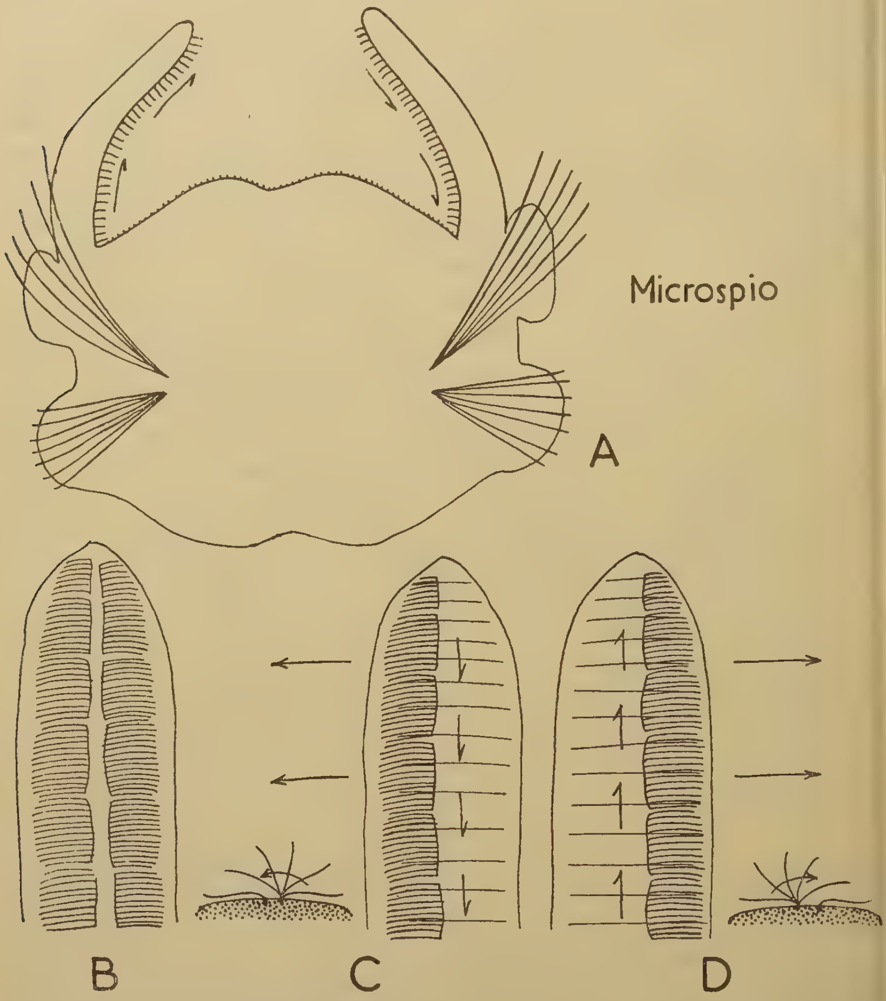

FIG. 7. A, transverse section of *Microspio mecznikowianus* showing movement of waves when the dexioplectic cilia of the branchiae are beating away from the observer; B, inner surface of a branchia showing the two rows of cilia, immobilized in the initial position of the effective stroke; C, branchia in section and in surface view showing the left-hand row of cilia still motionless and the right-hand row beating with dexioplectic metachronism (the beating cilia are indistinguishable individually in surface view); D, the same, but with the other row of cilia active.

assumption that these waves are laeoplectic it can be deduced that the cilia are beating away from the observer towards the midline, and hence that i must be the inner demibranchs which are developing first, as is known to be the general rule in lamellibranchs. It would be interesting to apply such observations to *Xylophaga* and the Teredinidae, adults of which lack the inner demibranch (Purchon, 1941), though the characteristic opacity of the larval shell in these families would no doubt prove to be an obstacle.

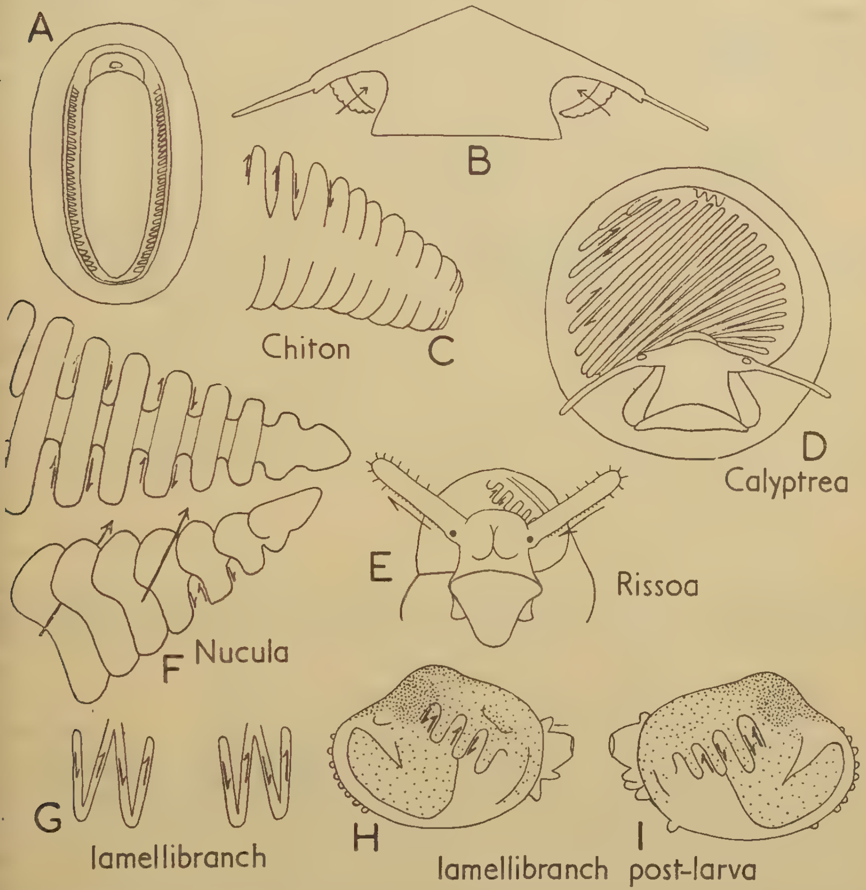

FIG. 8. Laeoplectic metachronism in some adult and post-larval Mollusca. A, *Chiton olivaceus* viewed ventrally, to show positions of ctenidia; B, *Chiton* in transverse section, to show movement of water currents drawn through the ctenidia by the lateral cilia; C, a ctenidium of *Chiton* viewed ventrally, showing the laeoplectic metachronal waves of the lateral cilia, which are beating away from the observer; D, *Calyptrea chinensis* viewed antero-ventrally, showing the greatly enlarged ctenidium and the waves of its lateral cilia, which beat away from the observer; E, *Rissoa soluta* viewed antero-ventrally, showing the waves produced by the cilia on the ctenidium and on the ventral surface of each tentacle, as they beat away from the observer; F, posterior ends of the paired ctenidia in *Nucula nitida*, viewed ventrally and from slightly to the left of the mid-line, showing waves produced by the lateral cilia, and currents produced by the lateral cilia of the right (lower) ctenidium (on the left ctenidium these cilia are beating directly away from the observer); G, diagrammatic cross-section of the ctenidia in a lamellibranch such as *Chlamys*, showing the movement of the waves produced by the lateral cilia, which presents a similar picture in both anterior and posterior views of the filaments; H, a eulamellibranch post-larva, from the left side, showing the movement of the waves produced by the lateral cilia of the rudimentary gill filaments; I, the same from the right side, to show that this movement is bilaterally asymmetrical.

Some gastropods, for instance *Rissoa soluta* (fig. 8, E), have tracts of cilia on the tentacles, which show laeoplectic waves.

The velum of larval Mollusca usually shows clearly diaplectic waves, which are laeoplectic in most molluscs, but dexioplectic in Eulamellibranchiata. These waves were clearly laeoplectic in gastropod larvae of 29 identified species, representing all orders except pulmonates, and in many other gastropod larvae which could not be identified.

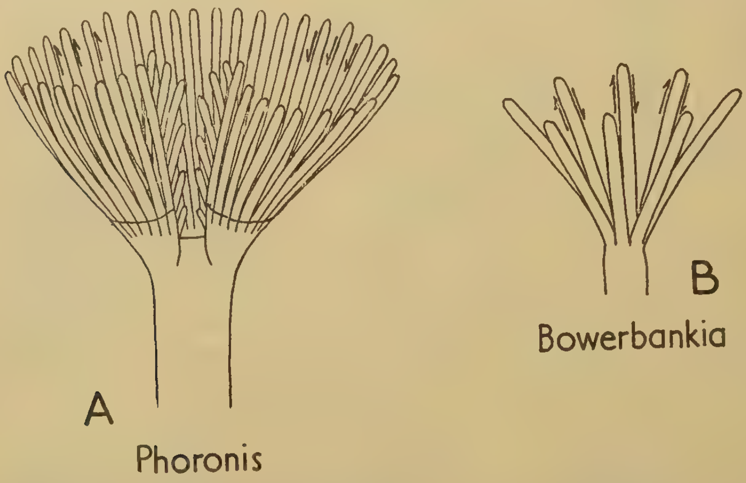

FIG. 9. Lophophores of A, *Phoronis*, with dexioplectic waves shown by the lateral cilia of the filaments, and B, the bryozoan *Bowerbankia*, with laeoplectic waves. In these forms the lateral cilia beat outwards.

Larvae of *Lepidochitona cinereus*, like those of most molluscs, rotate in what appears in apical view to be a clockwise direction. Their movement is unusually rapid, each rotation taking about 3 seconds. Their prototrochal waves are almost certainly laeoplectic, for they travel in the same direction as that of rotation, but at an even greater speed, moving through a complete circle in less than 2 seconds.

Larval oysters and mussels have laeoplectic metachronism on the velum. This agrees with the latest classification uniting these in the Anisomyaria, a group equivalent to the Eulamellibranchiata, veligers of which have clearly dexioplectic waves. No metachronal waves could be distinguished in trochophores of *Dentalium* or in veligers of the anisomyarian genera *Lima* and *Anomia*. Identification of larvae of Anisomyaria is considerably aided by observations of the velar waves and of the directions of rotation of the larvae (Knight-Jones, 1954).

Phoronidea (table 5, see p. 519)

Dexioplectic waves are produced by the ciliated girdles of actinotroch larvae (fig. 4) and by the lateral cilia of the adult tentacles (fig. 9).

Brachiopoda—Testicardines

Mr. Gareth Owen and Dr. R. B. Pike kindly sent living brachiopods from Scotland. Careful observation distinguished dexioplectic waves amongst the lateral cilia on the lophophore of *Terebratulina retusa*. The beat of these cilia was at right angles to the filaments, and from the inside towards the outside of the lophophoral loops. They were therefore important in causing a circulation of water, with incurrent lateral streams and an excurrent median stream, similar to that described by Richards (1952) in *Neothyris*, and by Orton (1914) in *Crania*. Richards did not observe any special activity of the lateral cilia.

Brachiopoda—Ecardines

Diaplectic metachronism could not be distinguished in larvae or adults of *Lingula* (Purchon, 1953) or in adults of *Crania anomala*.

Bryozoa (table 5, p. 519)

Laeoplectic waves are produced by the larval girdles (fig. 3) and by the lateral cilia of adult tentacles (fig. 8).

No metachronal waves could be distinguished in either larvae or adults of Endoprocta.

Rotifera (table 6, p. 520)

Dexioplectic waves are characteristic of the Bdelloidea and Melicertida (= Flosculariacea, Harring, 1913), laeoplectic waves of the Ploima (p. 516).

CONCLUSIONS OF SYSTEMATIC INTEREST

The results of the foregoing survey are summarized in table 7 (p. 520). In view of the facts that *Chaetopterus* is one of the most specialized of the polychaetes, and that the Mollusca form one of the most specialized groups of the Spiralia (groups showing spiral cleavage), it is highly probable that the primitive direction of diaplectic metachronism in the Spiralia was dexioplectic. Clearly this direction is reversed by mutation only at rare intervals.

The waves on the velum of eulamellibranch larvae probably represent a secondary reversion to the dexioplectic type for three reasons. First, laeoplectic metachronism is of general occurrence in Mollusca, being found in Amphineura, Gastropoda, and Lamellibranchiata. Secondly, the Anisomyaria, which have larvae with laeoplectic waves, have primitive features, including a larval hinge like the *Nucula* hinge, whilst the Eulamellibranchiata, on the other hand, form a compact and highly evolved group. Thirdly, the Eulamellibranchiata themselves show laeoplectic waves on their ctenidia, and the combination, in a single animal, of the two types of diaplectic metachronism, is a rare feature and so probably a secondary one. Although the most primitive groups of the Spiralia have dexioplectic waves, it therefore seems probable that the Precambrian ancestor of all the molluscs had already changed to laeoplectic waves, and that since then the veligers of eulamellibranchs have changed back again.

The current division of the Rotifera into Digononta and Monogononta (table 6, p. 520), seems unsound, because it unites the Melicertida with the Ploima and divides them from the Bdelloidea. The question whether the gonads are paired or single is of doubtful systematic importance, since reduction may easily have occurred more than once in the evolution of these minute forms. The direction of diaplectic metachronism seems a more reliable guide to affinity, since it is of no adaptive importance and is probably bound up with intimate cell structure (p. 517). The Bdelloidea and Melicertida somewhat resemble each other in the form of the corona and mastax, so if further observations confirm the grouping set out in table 6, the Bdelloidea and Melicertida might well be linked as sub-orders of the Dexioplectica, a new order of rotifers, the members of which show dexioplectic metachronism. The remaining rotifers, which show either laeoplectic metachronism, or extreme reduction or modification of the ciliary apparatus, would fall into the three orders—Ploima, Seisonidea, and Collothecacea.

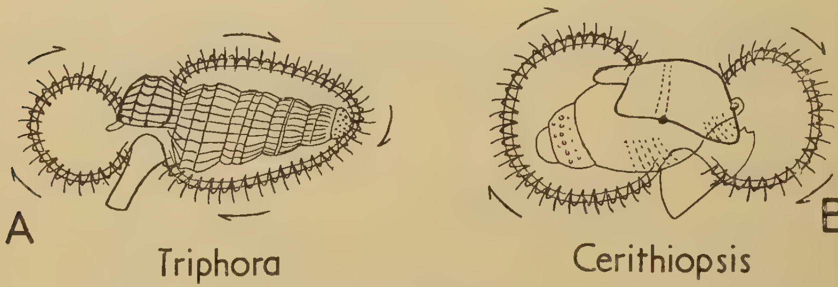

FIG. 10. Movement of metachronal waves in veligers, A of the *situs inversus* species of gastropod, *Triphora perversa*, and B of the normal species, *Cerithiopsis tubercularis*.

PROBABLE LOCATION OF THE FACTOR DETERMINING WHETHER DIAPLECTIC METACHRONISM IS DEXIO- OR LAEOPLECTIC

Triphora perversa (fig. 10) is a gastropod showing complete *situs inversus* of the type which is known to be associated in certain pulmonates with reversed spiral cleavage. Its metachronal waves nevertheless travel round the velum in the same direction as in a normal gastropod. Similarly, *Spirorbis pagenstecheri* and *Spirorbis borealis* show contrasting morphological asymmetries, as a result of which they undergo dextral and sinistral coiling respectively, yet the direction of metachronism is the same in larvae of both species (table 3, p. 518).

Conversely, though metachronism is laeoplectic in gastropods and *Chaetopterus*, and dexioplectic in polyclads, nemertines, and most polychaetes, the direction of spiral cleavage is well known to be remarkably constant throughout all groups of the Spiralia. Cleavage in *Chaetopterus* is in the usual direction (Mead, 1897).

It may be concluded that the asymmetry associated with diaplectic metachronism is independent of the familiar morphological asymmetries of animals. This independence is analogous with that shown by the spiralling of the sperm tail in *Limnaea*, which is in a constant direction in sperm from both normal and *situs inversus* individuals (Selman and Waddington, 1954). Evidently the direction of diaplectic metachronism is not determined by gross anatomy but probably by the micro-anatomy of the ciliated cells. It is likely to depend on some stereochemical peculiarity of the ciliary basal apparatus.

I am much indebted to the Royal Society for a grant from the Browne Fund; to the British Association for the use of their table at Naples; to Dr. R. Dohrn and his staff, and particularly to Dr. G. Bacci, for the excellent facilities which they provided at the Stazione Zoologica; to Professor F. W. Rogers Brambell, F.R.S., Dr. D. J. Crisp, and Sir James Gray, F.R.S., for their valuable interest and advice; to Dr. D. P. Wilson for much information and for the photographs; and to Dr. C. Burdon-Jones and the many other persons whose observations on larval cilia have been recorded in the tables.

TABLE 1

Examples of ciliated epithelia showing antiplectic metachronism

	Species observed
Swimming combs of Ctenophora	*Pleurobrachia pileus*
Antero-dorsal cilia of Turbellaria	*Dugesia tigrina* *Crenobia alpina*
Ventral groove in Serpulidae	*Pomatoceros triqueter* *Spirorbis borealis*
Tentacles and body in larval Chaetopteridae	*Chaetopterus variopedatus*
Food-collecting sulcus bordering velum in larval Gastropoda	*Nassarius incrassatus*
Frontal surface of ctenidium in Lamellibranchiata	*Cardium edule*
Collar and trunk epidermis of Enteropneusta	*Saccoglossus cambrensis*

TABLE 2

Protochordata in which dexioplectic metachronism was observed

HEMICHORDATA: *Saccoglossus cambrensis, S. horsti, Glossobalanus minutus*—lateral cilia of gill slits.
Saccoglossus horsti, S. pusillus, and *Tornaria ritteri* (Ritter and Davis, 1904), *Balanoglossus clavigerus, Glossobalanus sarniensis* (Wilson, 1952)—larval telotroch.

UROCHORDATA:
ASCIDIACEA: *Ascidia californica* (McGinitie, 1939), *Ascidiella aspersa, Ciona intestinalis, Perophora listeri, Diplosoma listerianum*—gill slits.
THALIACEA: *Doliolum mulleri* (Fedele, 1923)—gill slits.
LARVACEA: *Fritillaria urticans* (Fol, 1872)—gill slits.

CEPHALOCHORDATA: *Amphioxus lanceolatus* (Müller, 1842)—gill slits and wheel organ.

TABLE 3

Primitive Spiralia in which dexioplectic metachronism was observed

POLYCLADIDA: polyclad B* (Müller, 1850), *Thysanozoon brocchii.**

NEMERTINEA: Pilidium larvae* of several species.

SIPUNCULOIDEA: *Sipunculus nudus.**

ARCHIANNELIDA: *Dinophilus taeniatus.*†

POLYCHAETA ERRANTIA:

APHRODITIDAE: *Harmothoë imbricata, Malmgrenia castanea,*† *Halosydne gelatinosa,*† *Scalisetosus pellucidus,*† *Acholoë astericola* (Davenport, 1952), *Sthenelais limicola,*† *Sthenelais boa.*†
PHYLLODOCIDAE: *Phyllodoce maculata, Phyllodoce lamelligera,*† *Phyllodoce mucosa,*† *Eulalia viridis,** *Eulalia marginata.**
HESIONIDAE: *Kefersteinia cirrata,*† *Magalia perarmata.*†
SYLLIDAE: *Syllis prolifera,*† *Eusyllis blomstrandi,*† *Odontosyllis gibba, Autolytus aurantiacus.*†
NEREIDAE: *Nereis pelagica,** *Nereis virens** (Burdon-Jones, 1953).
NEPHTHYDIDAE: *Nephthys caeca,*† *Nephthys hombergi.*†
EUNICIDAE: *Eunice vittata,*† *Staurocephalus rudolphii,*† *Ophryotrocha puerilis.*

POLYCHAETA SEDENTARIA:

ARICIIDAE: *Nainereis laevigata,*† *Aricia cuvieri,*† *Scoloplos armiger.*†
SPIONIDAE: *Nerine cirratulus, Nerine bonnieri,*† *Nerine foliosa,** *Scolelepis fuliginosa, Microspio mecznikowianus, Prionospio malmgreni,** *Polydora antennata, Polydora ciliata.*
DISOMIDAE: *Poecilochaetus serpens.**
PARAONIDAE: *Aricidea jeffreysii.*†
CIRRATULIDAE: *Cirratulus cirratus** (Burdon-Jones, 1953).
CHLORHAEMIDAE: *Flabelligera affinis,*† *Stylarioides eruca.*†
OPHELIIDAE: *Ophelia bicornis** (Wilson, 1952), *Ammotrypane aulogaster.*†
CAPITELLIDAE: *Capitella capitata,** *Notomastus latericeus.*†
ARENICOLIDAE: *Arenicola marina** (Howie, 1952).
SABELLARIIDAE: *Sabellaria spinulosa,*† *Sabellaria alveolata.*
AMPHICTENIDAE: *Pectinaria koreni.**
AMPHARETIDAE: *Ampharete grubei.*†
TEREBELLIDAE: *Polymnia nebulosa.**
SABELLIDAE: *Dasychone lucullana.*†
SERPULIDAE: *Salmacina dysteri,** *Pomatoceros triqueter** (Burdon-Jones, 1953), *Spirorbis borealis,** *Spirorbis pagenstecheri.**

Metachronism was laeoplectic in:

CHAETOPTERIDAE: *Chaetopterus variopedatus.**

* Only larvae observed. † Only adults observed. The absence of an asterisk or a dagger indicates that metachronism was observed in both larvae and adults.

TABLE 4

Mollusca

The lateral cilia of the ctenidia showed laeoplectic metachronism in:

AMPHINEURA: *Chiton olivaceus.*

GASTROPODA: *Fissurella nubecula, Rissoa parva, Rissoa soluta, Calyptraea chinensis.*

LAMELLIBRANCHIATA: *Nucula nitida, Mytilus edulis, Ostrea edulis* (Walne, 1952), *Lasaea rubra, Cardium edule, Chione fasciata, Cultellus pellucidus.*

The velar cilia showed laeoplectic metachronism in:

AMPHINEURA: *Lepidochitona cinereus.*

GASTROPODA:

PROSOBRANCHIATA: *Patella coerulea, Patina pellucida, Lacuna vincta, Littorina littorea, Rissoa soluta, Hydrobia ulvae, Crepidula fornicata, Trivia monacha, Cerithiopsis tubercularis, Triphora perversa, Balcis devians, Nassarius reticulatus, Nassarius incrassatus, Mangelia striolata, Nucella lapillus, Urosalpinx cinerea* (Hancock, 1953).

TECTIBRANCHIATA: *Philine aperta, Aplysia punctata* (Burdon-Jones, 1953), *Aplysia depilans.*

PTEROPODA: *Limacina inflata, Clio (Creseis) acicula.*

NUDIBRANCHIATA: *Limapontia depressa* and *Elysia viridis* (Burdon-Jones, 1953), *Acanthodoris pilosa, Adalaria proxima, Archidoris britannica, Idulia coronata, Facelina curta, Tergipes despectus* (Norris, 1952), *Cratena aurantia.*

LAMELLIBRANCHIATA:

ANISOMYARIA: *Ostrea edulis, Mytilus edulis, Mytilus galloprovincialis, Modiolaria marmorata.*

The velar cilia showed dexioplectic metachronism in:

EULAMELLIBRANCHIATA: *Kellia suborbicularis, Mysella bidentata, Montacuta ferruginosa*, several Tellinidae, *Abra prismatica, Spisula elliptica, Petricola lithophaga, Cardium edule, Cultellus pellucidus, Mya arenaria, Zirphaea crispata, Teredo pedicellata* (Isham, 1951), *Teredo navalis.*

No metachronal waves could be distinguished in trochophores of *Dentalium dentale* or in veligers of the anisomyarian forms *Lima hians* and *Anomia ephippium.*

TABLE 5

Dexioplectic metachronism is found in:

BRACHIOPODA TESTICARDINES: *Terebratulina retusa.**

PHORONIDEA: *Phoronis hippocrepia, Phoronis psammophila.*

Laeoplectic metachronism is found in:

BRYOZOA: Cyphonautes larvae of several species.

CTENOSTOMATA: *Alcyonidium gelatinosum, Alcyonidium hirsutum,* Flustrella hispida, Bowerbankia imbricata, Zoobotryon pellucidum** (Cori, 1937).

CHEILOSTOMATA: *Bugula avicularia, Bugula plumosa, Bugula flabellata, Lepralia pallasiana.*

CYCLOSTOMATA: *Crisia cornuta.**

PHYLACTOLAEMATA: *Plumatella repens.**

Diaplectic metachronal waves could not be distinguished in:

BRACHIOPODA ECARDINES: *Crania anomala** (Pike, 1953), *Lingula sp.* (Purchon, 1953).

ENDOPROCTA: *Pedicellina cernuum, Loxosoma singulare.*

* Observations on adults only. In species not marked with an asterisk the cilia of the larval girdles were observed as well as the lateral cilia of the adult tentacles.

Table 6

In Rotifera, the metachronal waves of the trochal disk may be either dexioplectic (anticlockwise when viewed anteriorly) or laeoplectic (clockwise) as follows:

Dexioplectic	*Laeoplectic*
DIGONONTA	
BDELLOIDEA	
Philodina citrina	
Rotaria macroceros	
MONOGONONTA	
MELICERTIDA	PLOIMA
*Conochilus unicornis** (Gossler, 1952)	*Asplanchna priodonta** (Gossler, 1952)
Melicerta ringens (Cubitt, 1870)	*Epiphanes* (*Hydatina*) *senta*
Ptygura (*Oecistes*) *ptygura*	*Diaschiza semiaperta*
Ptygura (*Oecistes*) *crystallina*	*Mytilina* (*Salpina*) *mucronata*
	Platyias (*Noteus*) *quadricornis*
	Synchaeta sp. and several other *sp. incert.*

No ciliary activity could be observed in *Collotheca* (*Floscularia*) *regalis*.

* Previous records (Gossler, 1950) of the reverse direction of metachronism in these species, were due to a correction having been applied for optical inversion (Gossler, 1952). No such correction should be applied.

Table 7

Dexioplectic		*Laeoplectic*
Platyhelminthes		
Nemertini		
Sipunculoidea		
Archiannelida		
Polychaeta generally	⟶	Chaetopterus
Ancestral Spiralia?	⟶	Mollusca generally
Eulamellibranch veligers	↙	
Phoronidea		Bryozoa
Brachiopoda		
Bdelloidea and Melicertida		Ploima
Protochordata		

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Volume 95. Part 4

CONTENTS

Barer, R., & Joseph, S. Refractometry of Living Cells. Part I. Basic Principles 399

Ross, K. F. A. The Changes of Water Distribution in Cytoplasm and Nuclear Sap during Division as Indicated by Changes in their Refractive Indices 425

Davies, H. G. The Action of Fixatives on the Ultra-violet-absorbing Components of Chick Fibroblasts 433

Edwards, R. L. The Effect of Dieton Egg Maturation and Resorption in *Mormoniella vitripennis* (Hymenoptera, Pteromalidae) 459

Worst, J. A Micro-injection System 469

Allen, J. A. On the Structure and Adaptations of *Pandora inaequivalvis* and *P. pinna* 473

Weddell, G., Pallie, W., & Palmer, E. The Morphology of Peripheral Nerve Terminations in the Skin 483

Knight-Jones, E. W. Relations between Metachronism and the Direction of Ciliary Beat in Metazoa 503

NOTICE TO CONTRIBUTORS inside front cover

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